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RESEARCH ARTICLE

Activity test of fruit and pomegranate seeds (*Punica granatum* l) as a hepatoprotector against white male Wistar rats

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Abstract

Introduction: White pomegranate (*P. granatum*) is a species of the Punicaceae which is thought as hepatoprotector based on its antioxidant secondary metabolite compoundst. **Aims:** The purpose of study was to determine the hepatoprotector activity of white pomegranate fruit and seeds ethanol extract on male white rats Wistar strain. **Methods:** This research is laboratory experimental which rats were grouped into 5 groups randomly. Normal, negative (given Carboxymethylcellulose sodium 1% orally), test dose 1,2,3 (given extract at a dose of 50 mg /200 g bodyweight of rat, 100 mg/200 g bodyweight of rat and 200 mg/200 g bodyweight of rat in Carboxymethylcellulose sodium 1% orally). Negative, test dose 1,2,3 induced with paracetamol 180 mg/200 g bodyweight of rat. **Results:** From the statistical analysis, it was found that a significant difference between the negative with test dose 1, 2, and 3. The best hepatoprotector activity was produced by the test dose 3 with the percentage reduction in AST levels 91.62% and ALT 90.20%.

Introduction

The liver is an organ that has the potential to experience damage due to various therapeutic chemicals and the environment because of its function in metabolic processes and the detoxification of chemicals that enter the body. The damage that occurs to the liver will disrupt metabolism in the body, causing homeostatic disorders (Akhlaghi & Bandy, 2009). Liver damage caused by acetaminophen, also known as paracetamol, occurs due to a metabolite of NAPQI (N-acetyl-p-benzoquinoneimine), which is highly reactive. Under normal circumstances, this reactive product quickly binds to the liver's glutathione levels, making it a non-toxic material. However, in a state of overdose, or continuous use that causes NAPQI production to continue to increase and is not proportional to glutathione levels, NAPQI binds to form macromolecules with liver cells resulting in liver cell necrosis, covalent binding levels that determine the level of binding to macromolecules in causing cell injury (Apriliani *et al.*, 2015). Hepatoprotectors are compounds or substances that can protect liver cells from toxic effects. Judging from the structure, compounds that are hepatoprotective include phenylpropanoid, coumarin, lignin, essential oils, terpenoids, saponins, flavonoids, organic acids, lipids, alkaloids and xanthines. Several natural antioxidant compounds such as flavonoids, terpenoids, and steroids have been pharmacologically studied to have hepatoprotective activity. The largest source of antioxidants in nature is the phenolic or polyphenol component, while the rest are nitrogen and carotenoid components (Atmani D *et al.*, 2015). Until now, there is no drug that specifically treats liver damage caused by drugs; therefore, it is necessary to conduct research to explore herbal medicines that can be used as hepatoprotectors. One of the herbal medicines being

explored is the fruit and seeds of the white pomegranate (*P. granatum*).

Pomegranate fruit and seeds are used to treat intestinal worms, and it is believed to have a hepatoprotective effect (Apriliani *et al.*, 2015). They also have antibacterial properties. Previous research has proven that the ethanol extract of red pomegranate at a dose of 500 mg/kg body weight (bw) of rats can inhibit liver damage because, at this concentration, the number of necrotic cells is low, there are no apoptotic cells, and the amount of fat degeneration is low (Isselbacher *et al.*, 2014). In this study, the researchers wanted to prove the activity of the white pomegranate fruit and seeds as hepatoprotectors.

Methods

Equipment

The equipment used included macerator, powder making tools, test tube, Erlenmeyer, evaporator, pipette, oral probe, micropipette, syringe 1 cc, scales, microcentrifugation and Effendrop tube.

Materials

The test materials used in this study included Simplicia of white pomegranate fruit and seeds, Diasys reagent kit consisting of AST and ALT reagents, 96% ethanol, 1% CMC, aqua dest, filter paper, paracetamol tablets, 2N HCl, acetone, powder boric acid, oxalic acid powder, ether, Mayer reagent, dragendroff reagent, NaOH, anisaldehyde-sulfuric acid or vanillin-sulfuric acid, Liebermann-Burchard, 1% $FeCl_3$, 1% gelatin.

Collection and determination of samples

The fruit and white pomegranate seeds were obtained from the Cilolohan area of the city of Tasikmalaya, and the determination was carried out in the Herbarium of the School of Life Sciences and Technology Bandung Institute of Technology.

Manufacture of Simplicia

The fruit and seeds of the white pomegranate (*P.granatum*) were collected first; then, wet sorting was carried out. The fruit and seeds of the white pomegranate (*P. granatum*) were cut into small pieces, then washed in running water until clean, then dried in an oven at 70°C. After drying, it was sorted again to separate it from other impurities. The fruit and pomegranate seeds then were dried in sunlight and covered with a black cloth with the dried fruit and seeds evenly pressed. The fruit and seeds of the dried white pomegranate (Punica granatum L) were then pulverised.

Extraction

Extraction was carried out by immersing 500 grams of white pomegranate fruit and seeds with 2L of 96% ethanol solvent. The solvent was changed three times.

Standardisaton of simplicia

Specific parameters used were organoleptic examination. Simplicia examination was conducted with four senses, including smell, taste, shape and colour of Simplicia.

Non-specific parameters: phytochemical screening

Phytochemical screening was conducted by examining the content of alkaloids, flavonoids, saponins, tannins and polyphenols, monoterpenes and sesquiterpenes, steroids and triterpenoids.

Preparation of test animals

A total of 25 male rats were acclimatized to the environment for seven days, and each cage was given husks and well maintained.

The classification of the test animals can be seen in Table I.

Table I: Classification of animals and treatments

Classification	Treatment
Negative	CMC 1% and Paracetamol 180 mg/200 g bw
Normal	Not treated
Test dose 1	White pomegranate fruit and seeds ethanol extract at a dose of 50 mg/200 g bw in CMC 1% and Paracetamol 180 mg/200 g bw
Test dose 2	White pomegranate fruit and seeds ethanol extract at a dose of 100 mg/200 g bw in CMC 1% and Paracetamol 180 mg/200 g bw
Test dose 2	White pomegranate fruit and seeds ethanol extract at a dose of 200 mg/200 g bw in CMC 1% and Paracetamol 180 mg/200 g bw

The rat blood serum was taken to measure the levels of aspartate transaminase (AST) and alanine aminotransferase (ALT). Blood was centrifuged at 2000 rpm for 15 minutes to obtain serum. A total of 100 µl of serum was mixed with 1000 µl of kit reagent. At room temperature, the mixture was measured on a photometer with a wavelength of 340 nm and a factor of 17,456 without incubation.

Statistical analysis

The data obtained were analysed for normality test, homogeneity test followed by one-way ANOVA test with a 95% confidence to identify significant differences between all test groups.

Results

Orgnoleptic examination

The organoleptic results showed a distinctive odour, sweet taste, and brown colour, which can be observed in both powders and extract forms (see Table II).

Table II: Results of the quality inspection of the Simplicia powder and crude extract

Examination	Simplicia powder	Simplicia extract
Color	Chocolate	Chocolate
Smell	Typical	Typical
Taste/shape	Sweet	Sweet

Phytochemical screening

The screening of phytochemical from the extract and powdered fruit and seeds of the white pomegranate (*P. granatum*) contained flavonoid, alkaloid, tannin, polyphenols, monoterpenes and sesquiterpenes (See Table III). This result coincides with earlier studies that state extract of fruit meat and seeds of white pomegranate contained flavonoids, alkaloids, and polyphenols (Apriliani, 2015).

The presence of flavonoids in the Simplicia of the fruit and seeds of the white pomegranate can be used as an antioxidant and hepatoprotector to protect the components of liver cells from free radicals produced by paracetamol. It will donate hydrogen atoms to free radicals so that they can inhibit and neutralise the occurrence of oxidation reactions. Flavonoids are active compounds included in antioxidant intermediates that act as hydrophilic and lipophilic antioxidants. The antioxidant mechanism of flavonoids is to capture ROS directly, prevent ROS regeneration and indirectly increase the antioxidant activity of cellular antioxidant enzymes (Akhlaghi & Bandy, 2019). Prevention of the formation of ROS by flavonoids is carried out in several ways, namely inhibiting the action of the enzymes xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, as well as chelating metals (Fe^{2+} and Cu^{2+}) so as to prevent redox reactions that can produce free radicals (Akhlaghi and Bandy 2019; Atmani *et al.* 2015).

Table III: Results of phytochemical screening of pomegranate seed powder and extract

Sample	Alkaloid	Flavonoids	Saponins	Tannins and Pholiphenol	Steroids and Triterpenoid	Quinone	Seskuiterpenoids and Monoterpenoid
Seed powder	-	+	-	+	-	+	+
Seed extract	-	+	-	+	-	+	+

Information : (+) = detected (-) = not detected

Measurement of AST and ALT levels

Transaminase is an amino acid catabolism process that involves the transfer of an amino group from one amino acid to another. In this transaminase reaction, the amino group of an amino acid is transferred to one of the three keto compounds, namely pyruvic acid, oxaloacetic acid and a-ketoglutarate, so that this keto compound is converted into amino acids while the original amino acids are converted into keto acids. Transaminase enzymes in serum do not have a function as an enzyme because, in the serum, there is no coenzyme and the right substrate. Transaminase enzymes present in serum are an indicator of tissue damage in certain diseases (Lotito SB, 2000).

The liver itself is able to secrete transaminase enzymes when the cells are damaged. High transaminase levels usually indicate liver abnormalities and necrosis. These enzymes enter the bloodstream. Transaminases are sensitive indicators of damage to liver cells.

Aspartate Transminase (AST)

Aspartate Transaminase (AST) is an enzyme found in heart muscle and liver, while moderate concentrations are found in skeletal muscle, kidney and pancreas. Low concentrations are also found in the blood; unless there is cellular injury, large amounts are released into the circulation. In cardiac infarction, AST will increase for a period of ten hours and reach its peak 24-48 hours after the infarction. AST will return to normal after 4-6 days if no additional infarction occurs. AST levels are usually compared with levels of other heart enzymes, such as CK (creatine kinase) and LDH (lactate dehydrogenase).

In liver disease, the levels will increase ten times more and remain so for a long time (Lotito SB, 2000).

AST serum is generally measured via photometry or spectrophotometry using a photometer or spectrophotometer or using a chemistry analyser. Reference values for AST in males are 0-50 U/L, while for females, it is 0-35 U/L (Lotio SB, 2000).

The AST level and the percentage decrease of AST level in the test groups can be seen in Table IV and Figure 1, respectively.

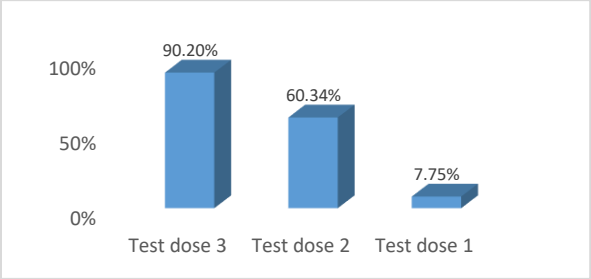

Figure 1: The percentage of decrease in AST level in test groups

Table IV: AST levels (mg/dL)

Rat number	Normal	Negative	Dose 1	Dose 2	Dose 3
1	22.22	86.21	66.24	28.51	6.27
2	27.51	73.11	72.22	24.22	8.13
3	32.11	74.13	54.21	36.18	7.29
4	31.17	82.18	63.31	29.04	6.32
5	23.42	83.42	70.11	31.36	5.43
Average± *SD*	**27.286±4.444**	**79.81±5.847**	**65.218±7.050**	**29.862±4.373**	**6.688±1.040**

Alanine Amino Transferase (ALT)

This enzyme catalyzes the transfer of amino groups like alanine and alpha-ketoglutarate acid. It is abundant in hepatocytes, and its concentration is relatively low in other tissues. Normal blood levels are 5-35 U/L, and ALT is more sensitive than AST (Lotito SB, 2000).

ALT and AST serum levels are elevated in almost all liver diseases. The highest levels are found in association with conditions causing extensive liver necrosis, such as severe viral liver hepatitis, toxin-induced liver injury, or prolonged circulatory collapse. A lower increase was found in mild acute hepatitis as well as in localized and diffuse chronic liver disease (Podolsky & Isselbacher, 2000). Levels drop suddenly in acute illness, indicating that the remaining source of the enzyme is depleted. If the damage by inflammation of the liver is only minor, ALT levels rise earlier and faster than AST levels.

In general, the ALT levels are higher than AST for acute liver parenchymal damage, while in the chronic process, it is the opposite (Lotito SB, 2000). Results of the ALT and AST measurements can be seen in tables 3 and 4.

The ALT level and the percentage decrease of ALT level in the test groups can be seen in Table V and Figure 2, respectively.

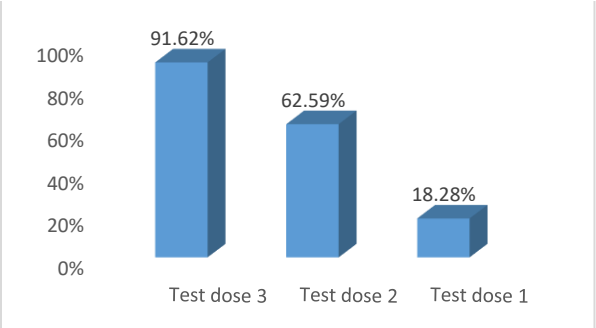

Figure 2: The percentage of decrease in ALT level in test groups

Table V: ALT levels (mg/dL)

Rat number	Normal	Negative	Dose 1	Dose 2	Dose 3
1	34.27	90.11	88.42	32.26	10.18
2	27.73	92.42	81.18	39.18	8.13
3	39.57	88.16	72.42	37.15	7.26
4	27.11	72.36	80.11	28.21	6.23
5	23.42	76.43	63.42	29.57	9.32
Average± *SD*	**30.42±6.436**	**83.896±8.92**	**77.4±9.524**	**33.274±4.751**	**8.224±1.272**

From Tables IV and V, it can be seen that paracetamol induction can increase AST and ALT levels, whereas, in the negative group, AST and ALT levels are greater when compared to the normal group.

Statistical analysis of AST and ALT levels

The test results were analyzed, including the normality test, homogeneity, ANOVA, and LSD test. Based on the Shapiro-Wilk test, it was found that the five treatment groups had AST and ALT data that were normally distributed ($p > 0.05$). In the homogeneity test using the Levene test, the results show that the data is homogeneous at AST ($p = 0.77$) and at ALT ($p = 0.11$). To see the difference in AST and ALT levels between treatment groups, a One Way Anova test was performed with the results of a significant difference ($p < 0.05$). Then the LSD test was carried out to see which group had a significant difference in Table VI.

From Table VI, it can be seen that there is a significant difference between the normal group and the negative group. The presence of a large dose of paracetamol in negative group adducts in liver proteins prior to hepatotoxicity is mainly dependent on P450-catalysed oxidative biotransformation to N-acetyl-p-benzoquinone imine (NAPQI) (Bessems, 2001). The negative group is significantly different from test doses 1, 2 and 3. This means that test doses 1, 2 and 3 are provided significant and effective hepatoprotection activity compared to the negative group. Test dose 3 was significantly different from normal, negative test dose 1 and test dose 2. This means that the dose of 3 (200mg / 200gram rat BW) is effective because the levels are close to normal levels. With a decreasing percentage of AST 91.62% and ALT 90.20%. In another study, AST and ALT levels were also lower than the negative control with pomegranate extract of 250 mg/kg BW and 500 mg/kg BW (Apriliani D, 2015).

Table VI: LSD test results levels of AST and ALT

Rat ID	Normal	Negative	Dose 1	Dose 2	Dose 3
Normal	-	SD	SD	NSD	SD
Negatie	NSD	-	SD	SD	SD
Test Dosage 1	NSD	NSD	-	SD	SD
Test Dosage 2	NSD	NSD	NSD	-	SD
Test Dosage 3	SD	SD	SD	SD	-

Description: SD : Significantly different ($p < 0.05$) ; NSD : Not significantly different ($p > 0.05$)

Conclusion

Based on the results of the study, the effect of giving ethanol extract of white pomegranate fruit and seeds (Punica granatum L) on Wistar male white rats induced by paracetamol can reduce AST and ALT levels. The best reduction was produced by the test dose group 3 (200mg / 200 g BW of rat), with a decreasing percentage of AST 91.62% and ALT 90.20%.In further testing, it is recommended to perform liver histopathology, and it is necessary to conduct research with another activity testing as a hepatoprotector.

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