Unripe Papaya (Carica papaya L.) Aqueous Extract (UPAE) for the acute toxicity test on fatty liver changes

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Abstract. We investigated whether the oral ingestion of aqueous extract of unripe papaya fruit (UPAE), would also induce fatty liver changes by analysing the UPAE-treated mice qualitatively. This study used laboratory experiments which performed by a proposed (new) recommended method with 11 subjects of female mice which were administered with single oral doses of 0; 50; 200; 400; 800; 1,000; 1,500; 2,000; 3,000; 4,000; 5,000 mg/kg respectively. All these mice were euthanized after 24 hours of UPAE administration. Histopathological studies were conducted using liver samples which were stained by Haematoxylin-Eosin (HE). The UPAE did not cause death in all doses but did induce hepatic steatosis and steatohepatitis at 4,000 and 5,000 mg/kg dosages. Ballooning degeneration, necroinflammation and portal fibrosis revealed at 200–5,000 mg/kg. These findings suggest that the UPAE provoked the fatty liver changes at the highest dose administration. It may be obtained from a multiple hit process by the accumulation of the phytochemical compounds of UPAE.

1. Introduction

Papaya plants (Carica papaya L.) are a type of tropical plant that is used as traditional medicine. The fruits contain a lot of vitamins, enzymes, and other active compounds such as saponins, alkaloids, and terpenoids. Unripe papaya fruit contains more nutrients and secondary metabolites than the ripe one. Alkaloids, saponins, flavonoids have antibacterial, antioxidant and anti-inflammatory, and galactagogic effects. However, the inappropriate use of the papaya fruit may cause adverse effects [1]. Development of research on medicinal plants needs to be done so that plants can be used for human medicine. Toxicity tests are needed to determine the level of safety and side effects of medicinal plants [2]. Herbal remedies which are commonly used for self-medication, drugs and toxins represent potential hepatotoxic agents because of the many idiosyncrasies and unpredictable toxicity. It should always be considered in the pathology of specimen evaluation [3].

The liver is the primary organ that is essential for drug metabolism and the major point organ of drug-induced injury. The most frequent pathologic manifestations of drug-induced hepatotoxicity, such as fatty liver changes (steatosis), steatofibrosis (fatty liver and fibrosis), and steatohepatitis (fatty liver with inflammation or necrosis and fibrosis) that can happen simultaneously or individually. The macro
vesicular steatosis is the more indolent form of steatosis. Steatohepatitis is associated with specific pathologic lesions in the liver, which include various amounts of macro vesicular steatosis, hepatocellular injury (most commonly seen in ballooning), and inflammation in the hepatic lobes or portal tracts, or both [3,4]. Nonalcoholic fatty liver diseases (NAFLD) affects all ages and ethnicities, and is the second leading cause of death in the general population [5,6]. At present, the high prevalence and negative pathological consequences of NAFLD represent a significant economic burden for many countries. However, up to now, there is no effective procedure to treat the disease [7,8]. Hence the present study is designed to assess the fatty liver changes in mice that are exposed to oral administration of unripe papaya aqueous extract.

2. Method
The experimental study was conducted at the Medical Biology Laboratory, Medical Faculty of Universitas Islam Bandung. This research is an experimental study using female mice as experimental animals from the Laboratory of Pharmacology, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java. This study has obtained ethical clearance from Universitas Islam Bandung Ethics Committee with the ethical approval number: 095/Komite Etik FK/III/2017.

2.1. Preparation of UPAE
Fresh unripe papaya fruits were harvested from a local plantation in Leles, West Java, Indonesia. It was authenticated at the herbarium of the Botany Department in the Bandung Institute of Technology. The fruit was unpeeled, approximately 2.5-3 months in age with a green papaya peel and a white color of both flesh and seeds. It was cut into small pieces and then extracted with 5 liters of water at room temperature for 72 hours and then concentrated to be dry in vacuum on a rotary evaporator to obtain the crude aqueous extract.

2.2. Preparation the experimental subject
Eleven female mice aged between 6-8 weeks, weighing 25-30 g, are obtained from the Medical Faculty of Padjadjaran University Bandung and were housed in plastic mice cages in groups of four mice per cage, in a room temperature of 25 ± 2°C for 12 hours in natural light and 12 hours in darkness with free access to tap water and dry mice pellets. They were allowed to acclimatize for seven days before the experiments.

2.3. Acute toxicity test of UPAE
The proposed recommended method that is new was selected in this toxicity test study with a single oral dose, used at 0; 50; 200; 400; 1,000; 1,500; 2,000; 3,000; 4,000; 5,000 mg/kg each for 24 hours [9]. This study used a pure experimental design in vivo with a simple random sampling technique.

2.4. Observation of liver preparation
After the animals were sacrificed, the mice liver was identified and carefully dissected out. The livers rinsed in a normal saline and sections were extracted from them. The liver tissue section was fixated in a 10% formol-saline, dehydrated with a 100% ethanol solution and embedded in paraffin. They were processed into 5 µm thick sections and stained with hematoxylin-eosin (HE) then observed under a light microscope, The Olympus CX23 LED Tokyo-Japan, with 100x and 400x magnification. The picture was captured by an Optic lab microscope camera and Image Raster tools were used for picture identification.

3. Results
We observed that the mice which were administrated by UPAE showed ballooning degeneration at 50 – 5,000 mg/Kg range dosages. Therefore, the necroinflammation and fibrosis were found at 200 – 5,000 mg/kg. The UPAE did induce hepatic steatosis and steatohepatitis at 4,000 and 5,000 mg/kg dosages (Table 1).
Table 1. Unripe Papaya Aqueous Extract (UPAE) acute toxicity test on fatty liver changes.

| Doses (mg/Kg) | Steatosis                      | Steatohepatitis  | Fibrosis           |
|--------------|--------------------------------|-----------------|--------------------|
|              | Macro-Vesicular | Micro-Vesicular | Ballooning Degeneration | Necro-inflammation |
| Control      | -                 | -               | -                  | -                 |
| 50           | -                 | -               | +                  | +                 |
| 200          | -                 | -               | +                  | +                 |
| 400          | -                 | -               | +                  | +                 |
| 800          | -                 | -               | +                  | +                 |
| 1000         | -                 | -               | +                  | +                 |
| 1500         | -                 | -               | +                  | +                 |
| 2000         | -                 | -               | +                  | +                 |
| 3000         | -                 | -               | +                  | +                 |
| 4000         | +                 | -               | +                  | +                 |
| 5000         | +                 | -               | +                  | +                 |

The photomicrographs of liver tissue sections from control and experimental mice stained with HE are shown below. The tissue sections of the experimental animals were inherently abnormal when compared with the control sections (figure 1 and 2).

Figure 1. The histopathological examination of liver sections: control mice (a) and steatosis lesion were observed on experimental mice (b, c).

Plate a (×100) are sections of liver from control mice treated with normal saline and stained with HE. On the other hand, plates b and c (×100) were sections of mice liver that received 4000 mg/kg and 5000 mg/kg respectively that showed the significantly abnormal typed macro vesicular steatosis. It is characterized by the presence of one or more large intracytoplasmic fat droplets that displace the hepatocellular nucleus to the peripheral of the cell. The further histopathological observations are shown below (figure 2).
Figure 2. Representative histological section of liver in the UPAE acute toxicity testing: ballooning degeneration (a), (b); necroinflammation (c), (d); and fibrosis (e), (f).

Plates a, c, and e (×100) were sections of mice liver that received 3000 mg/kg, 4000 mg/kg, 400 mg/kg respectively of the UPAE. Meanwhile, plates b, d, and f (×400) were sections of the liver of mice that received 1000 mg/kg, 2000 mg/kg, 200 mg/kg respectively of the UPAE. The sign of the fatty liver changes is found as ballooning degeneration revealed at 50 – 5,000 mg/kg range dosages. Furthermore, necroinflammation and portal fibrosis are observed at 200 – 5,000 mg/kg.

4. Discussion
It is reported that the safety assessment in experimental animals, of both medicine and non-medicine active chemicals, had caused toxicity in humans. The liver is one of the important organs of metabolism, detoxification, storage of xenobiotic and drugs. The phytochemical analysis of the aqueous extract of *Carica papaya L.* showed the presence of alkaloid, saponin, triterpenoid, flavonoids, quinone, and tannins [10]. The result of acute oral toxicity (LD50) of the unpeeled UPAE was found to be above 5000 mg/kg of body weight [11] and 2520 mg/kg of body weight of peeled UPAE [12].

This study found that UPAE at the highest dosage (4,000 -5,000 mg/kg) caused macro vesicular steatosis and revealed the sign of steatohepatitis. Hepatotoxicity occurs in most of those who are exposed and starts soon after some threshold for the toxicity reached. It could be intrinsic or idiosyncratic hepatotoxicity. Intrinsic hepatotoxicity is predictable, dose-dependent, and specific when consumed in
certain quantities which occurs by the damage of cells and organelles, toxin activation, or mechanisms that involves the immune system. Nevertheless, idiosyncratic hepatotoxicity involves unpredictable reactions depending on the individual genetic variation of drug metabolism and also environmental factors. Hepatic steatosis, which corresponds to an accumulation of triglyceride in hepatocytes, results from an imbalance between the delivery of free fatty acids and endogenous lipogenesis and fatty acid disposal through oxidation and packaging of esterified fatty acids or triglycerides for export by Apo lipoproteins as very-low-density lipoproteins [4].

Drug-induced macro vesicular steatosis might be the only histologic abnormality, or might be associated with varying degrees of micro vesicular steatosis, thereby resembling alcohol-induced steatosis. A high intramitochondrial concentration of protonated forms which inhibit which β-oxidation, might cause steatosis and lead to the mitochondrial formation of reactive oxygen species (ROS). It triggers steatohepatitis by lipid peroxidation, cytokine release, and fas ligand induction [3,13]. All of these mechanisms could lead to hepatocytes death, fibrosis, and chemotaxis of the neutrophils. This experiment found that the acute toxicity dosage could induce the sign of fatty liver changes. The ballooning degeneration is observed at all stages of the dosage. Necroinflammation and mild fibrosis is showed at dosage ranges of 200-5,000 mg/kg body weight.

The pathogenesis of fatty liver and steatohepatitis is also included in the roles of altered gut microbiota, intestinal permeability, and lipopolysaccharide (LPS) exposure, differential macrophage activation in adipose tissue depots, innate immunity, and supporting activation by inflammation in obesity-related liver injury. For these reasons, the limitation is set to the utilization of ingredients containing tannins, in order not to impair productive animal performance [14].

From the present study, it is showed that the tissue section of the liver from control animals was mostly normal. However, there were fatty liver changes in the tissues of the animals that received various acute toxicity doses of the unpeeled UPAE. The present finding is slightly different with the previous reports on the biochemical and hematological response in Wistar albino rats exposed to UPAE at lower dosages (100 and 300 mg/kg of body weight) that had a normal histopathological observation on rat liver [15]. The peel of papaya contains many laticifers that produce copious latex in unripe ones. It is rich in secondary metabolites, especially alkaloids which are included in many papaya enzymes such as caricain, chymopapain, papain. Our study uses the unpeeled skin of unripe papaya fruit that may be the cause of the different result to the previous study.

5. Conclusion
In conclusion a high dose ingestion of unripe papaya aqueous extract causes fatty liver changes in the liver tissues which are studied with mice. However, conducting a similar study using a more significant number of samples using special staining such as Oil Red O may be necessary to confirm lipid accumulation. Furthermore, evaluation of the level of mitochondrial injury with in vitro or in vivo diagnostic studies can be conducted to assess the pathomechanism of fatty liver changes significantly.

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