Redefining the hepatoprotective potential of Javanese turmeric (Curcuma xanthorrhiza) Kombucha towards the diethylnitrosamine-induced hepatotoxicity of mice

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Kombucha towards the diethylnitrosamine-induced hepatotoxicity of mice

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Abstract

Purpose – This research intended the utilization of Javanese turmeric (0.4% w/v) as a kombucha substrate and analysis of its hepatoprotective activity, in comparison against nonfermented Javanese turmeric beverage (JTB) and black tea kombucha.

Design/methodology/approach – Forty-two healthy male Balb/c mice (two- to three-week-old, 20–30 g) were divided into six groups with seven replicates each. The treatments were normal diet, normal diet þ Javanese turmeric kombucha (JTK), normal diet þ diethylnitrosamine (DEN), DEN þ JTB, DEN þ JTK, DEN þ black tea kombucha. Kombuchas and JTB were given at 0.3 mL/20 g BW/d. DEN was induced intraperitoneally at a dose of 100 mg/kg. Observed biomarkers were blood serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) activity, serum malonaldehyde (MDA), as well as liver histology. Data were analyzed using analysis of variance.

Findings – Among DEN-induced groups, JTK significantly ($p < 0.05$) diminished the level of blood SGPT, SGOT and serum MDA. JTK also had lower blood SGPT (8.604 6 2.195 U/L) and serum MDA levels

1. Introduction

Chronic liver disease occurs after the liver is exposed to prolonged inflammation. This inflammation can be caused by oxidative stress, alcohol, viral infection, accumulation of cytoplasmic fats or autoimmune disease. Chronic liver disease may alter several biochemical pathways related to nutrients and result in great nutritional impact (Fortes, 2017). Patients with early stages could undergo transplantation, abrasion or resection. However, symptoms may be hard to detect during the early stages and can lead to delayed treatment (Zhang *et al.*, 2021). Therefore, prevention is needed at the early stages to ease the burden and arrest the expansion of the disease.

Diet and dietary constituents seem to play a hepatoprotective role (Elsharkawy *et al.*, 2022). Javanese turmeric is an Indonesian local rhizome that is mostly used to alleviate liver disease (Rahmat *et al.*, 2021). It contained xanthorrhizol, curcuminoid, flavonoid, ar-turmerone, α-turmerone, curcumene, bisacurone, curlone, lactone-germacrone and germacrone (Lukitaningsih *et al.*, 2020). Xanthorrhizol, the most abundant active compound found in Javanese turmeric, is known as a good anti-inflammation compound and exhibits the ability to reduce the activity of blood serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT), enzymes that are considered as liver damage markers (Oon *et al.*, 2015). However, these identified bioactive compounds are bound with the plant matrix and other components, therefore they have low bioavailability (Rahmat *et al.*, 2021). Owing to the fermentation process many of these bioactive compounds could be released and become readily bioavailable.

Kombucha is a tea beverage fermented by a symbiotic association of bacteria and yeast (SCOBY) (Miranda *et al.*, 2022). The bacteria and yeast in kombucha are known to produce hepatoprotective components such as glucuronic acid and *D-saccharic acid 1,4-lactone* (DSL). Glucuronic acid can enhance the detoxification of toxicants, increase polyphenol bioavailability and increase the solubility of steroids and vitamin D, whereas DSL may act as a hepatoprotective agent and antioxidant (Martínez-Leal *et al.*, 2018). Recent research on kombucha has focused on using other new materials as substrates (Salafzoon *et al.*, 2018; Zubaidah *et al.*, 2018; Zubaidah *et al.*, 2022). Thus, the objective of this research was to analyze the usage of Javanese turmeric as a kombucha substrate to enhance its hepatoprotective properties. The microbial activity during fermentation was expected to synergize with Javanese turmeric active compounds and enhance their hepatoprotective activity.

2. Materials and methods

2.1 Materials

Javanese turmeric of commercial maturity, commercial kombucha starter, black tea and cane sugar were obtained in Malang, East Java, Indonesia. Javanese turmeric was peeled, cleaned and cut into thin slices. The slices were then dried in a cabinet drier at 60°C for 5–6h and ground into a fine powder. Diethylnitrosamine (DEN) was obtained from the Tokyo Chemical Industry.

2.2 Kombucha and nonfermented solution preparation and analysis

Javanese turmeric solution (0.4% w/v) was made with 2 g of Javanese turmeric powder that was placed into teabags. The concentration was chosen as the optimal treatment after the analysis of total microbial cells, total titratable acidity, pH, total phenolic content and antioxidant activity by Zubaidah *et al.* (2022). Javanese turmeric powder was extracted in boiling water (500 mL) for 5 min, then sweetened with 10% sugar. The solution was put into sterile glass jars and cooled to room temperature (at 30°C). Kombucha starter (10% v/v) was added aseptically. The jars were covered with sterile cheese cloths and fermentation was carried out at room temperature for 12 days. As a control, black tea kombucha was prepared with the same method at a concentration of 0.4% (Zubaidah *et al.*, 2018). Nonfermented Javanese turmeric beverage (JTB) was prepared with the same concentration and procedure as Javanese turmeric kombucha (JTK) without the addition of a kombucha starter.

2.3 Animal experiment and analysis

Forty-two healthy male BalB/C mice (two- to three-week-old, 20–30 g) were obtained from Biomedic Laboratory, Medical Faculty of Muhammadiyah University, Malang. The mice were divided randomly into six groups with seven replicates each, with Group 1 (normal diet), Group 2 (normal diet þ JTK), Group 3 (normal diet þ DEN), Group 4 (DEN þ JTB), Group 5 (DEN þ JTK) and Group 6 (DEN þ black tea kombucha). Each group shared one housing and the bedding was changed every three days. Mice received exposure to light for 12 h/day. Mice were acclimated for seven days, weighed once every three days, and were given JTK, black tea kombucha and JTB (0.3 mL/20 g BW/day) regularly for three weeks. The mice accessed standard diet (BR2 commercial feed) and water ad libitum throughout the experiment.

Alongside the treatments, DEN (100 mg/kg) was then induced intraperitoneally once per week for two weeks. Mice were then incubated for one week while the treatments were still being given. The mice were then fasted for one day. Twenty-four hours after fasting, the mice were sacrificed using 0.2 mL ketamine injection (0.1 mg/g BW). Blood was withdrawn from the heart during scarification for SGPT and SGOT activity analysis. SGPT and SGOT activity were analyzed using commercial reagent kits (Biosystems S.A, Costa Brava 30, Spain Quality system certified: EN ISO 13485 and EN ISO 9001 standard). Malonaldehyde (MDA; nM/mL) concentration was analyzed in blood serum using the standard spectrophotometric method (Zubaidah *et al.*, 2023) with an MDA kit (Biotect Co, China, ISO 9001:2015, EU2577). The animal study was approved by the Brawijaya University Research Ethics Committee (Ethical Clearance No. 109-KEP-UB-2021).

2.4 Histopathological analysis

Histopathological analysis of liver cells was carried out according to Zubaidah *et al.* (2023). After scarification, livers were taken and fixed in formalin, then dehydrated by 50%–100% ethanol. The livers were dissected, fixed in 5 *m*m paraffin and stained using hematoxylin and eosin. The stained dissections were observed through a light microscope (40x, 100x and 400x) to analyze the existence of inflammatory cells and apoptotic bodies. Damaged cells were counted.

2.5 Statistical analysis

Data are presented as mean 6 standard deviation (SD) (n ¼ 7). The data were analyzed by analysis of variance, followed by Fisher's exact test at $p < 0.05$.

3. Result and discussion

3.1 E□ect of treatments on mice behavior

Mice that were given different treatments exhibit different behavior. Group 1 (normal diet) and Group 2 (normal diet þ JTK) showed normally active physical activity and normal appetite. In comparison, Group 3 (normal diet þ DEN) showed less physical activity and became lethargic. The group also showed a decrease in appetite. DEN is an acute liver toxin. DEN injured the liver and decreased appetite and food consumption (You *et al.*, 2021). The DEN-induced group that was also given JTK, black tea kombucha and JTB was more active compared to Group 3. This group also showed a higher level of appetite compared to Group 3. These might be caused by Javanese turmeric and black tea kombucha chemical components that could increase appetite and repair metabolism (Rahmat *et al.*, 2021; Jayabalan *et al.*, 2014). JTK, JTB and black tea kombucha restored the appetite of mice induced by DEN.

3.2 Serum glutamate pyruvate transaminase and serum glutamate oxalate transaminase enzyme activity

SGPT is an enzyme only present in the liver parenchyma cell cytosol thus its activity specifically indicates liver failure (Desiandura *et al.*, 2022). SGOT is an enzyme found in liver cells cytosol and mitochondria. It also exists in cardiac muscle, skeletal muscle, pancreas and kidney. When the liver was exposed to DEN, the cells lysed and released SGPT and SGOT into the blood circulation (Castro *et al.*, 2015). The evaluation of SGPT and SGOT revealed the hepatoprotective activity of kombucha. Low levels of SGPT and SGOT showed better hepatoprotective activity. The results of SGPT and SGOT activity measurement are shown in Table 1.

The lowest SGPT activity was found in the normal diet þ JTK group. The normal SGPT activity value of healthy mice is 5–35 IU/L (Hutagalung *et al.*, 2023). The SGPT activity of the normal diet group (71.8%) was still within range and lower than the normal diet group, which meant JTK enhanced healthy liver condition. The lowest SGOT activity was found in the normal diet group. The SGOT value of the normal diet group was within the normal range of 10–40 IU/L (Hutagalung *et al.*, 2023). The normal diet þ JTK group had significantly higher SGOT activity compared to the normal diet group, but still within the normal range. However, the high SGOT activity was not able to specifically indicate liver

Treatments	SGPT (U/L)	%decrease	SGOT (U/L)	%decrease
Normal diet	25.8^{ab} 6 9.4	0.0	14.6^{c} 6 11.0	0.0
Normal diet þ Javanese turmeric kombucha	7.3^{c} 6 1.2	71.8	23.2^{bc} 6 2.9	−59.1
Normal diet þ DEN	36.4^{a} 6 13.6	0.0^{d}	37.1^{a} 6 3.7	0.0^{d}
DEN þ Nonfermented Javanese turmeric beverage	15.2^{bc} 6 8.7	58.2^{b}	25.8^{abc} 6 4.7	30.4^{b}
DEN þ Javanese turmeric kombucha	8.6^{c} 6 2.2	76.4^{a}	21.2^{bc} 6 8.6	42.9^{a}
DEN þ black tea kombucha	18.5^{bc} 6 13.0	49.1^{c}	31.8^{ab} 6 5.0	14.3^{c}

Notes: SGPT ¼ serum glutamate pyruvate transaminase; SGOT ¼ serum glutamate oxaloacetate transaminase; DEN ¼ diethylnitrosamine; SGPT and SGOT decrease percentage of normal diet þ Javanese turmeric kombucha were calculated based on the SGPT and SGOT value of normal diet. Decrease percentage of DEN þ nonfermented Javanese turmeric, DEN þ Javanese turmeric kombucha, and DEN þ black tea kombucha was calculated based on the SGPT and SGOT value of normal diet þ DEN; Data is presented in means 6 SD; data followed by different letters shows a significant difference ($p < 0.05$)

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Table 1.
Activities of SGPT and SGOT in blood serum of mice (n ¼ 7)

damage as SGOT activity is also present in different tissues such as cardiac muscle, skeletal muscle, pancreas and kidney (Desiandura *et al.*, 2022).

The normal diet þ DEN group showed the highest SGPT and SGOT activity (Table 1). The SGPT activity of this group was higher than the normal range of healthy mice, whereas the SGOT activity approached the upper limits. This indicated that DEN was able to induce liver damage and cause cell lysis. DEN was able to accumulate free radicals in liver cells and impair enzymes involved in liver DNA reparation. The free radicals harmed DNA and other nucleophilic compounds and resulted in the development of cancer cells (Arboatti *et al.*, 2018). When the liver cells were damaged, SGPT and SGOT were released from the cells and circulated in the bloodstream, therefore the DEN þ normal diet group showed high SGPT and SGOT activities (Castro *et al.*, 2015).

DEN þ JTB group had 58.2% lower SGPT and 30.4% lower SGOT activity compared to the normal diet þ DEN group. Javanese turmeric contains various antioxidant and anti-inflammatory compounds such as xanthorrhizol, curcumin, calebin-A, phenolic compounds and triterpenoids. Antioxidants lower the number of free radicals and inhibit liver cytochrome P450 2E1 (CYP2E1), a gene responsible for the induction of microsomal oxidation that increases free radical production. This activity made liver cells produce the right amount of adenosine triphosphate (ATP) and ions, therefore creating a homeostatic condition. This condition decreased osmotic pressure and prevented cell membrane leakage. This activity prevented the release of SGPT and SGOT into the bloodstream (Puteri *et al.*, 2020).

Xanthorrhizol suppressed the phosphorylation of c-Jun N-terminal kinase in the mitogen-activated protein kinases signaling pathway (Oon *et al.*, 2015). The suppression prevented the transcription of cyclooxygenase (COX), inducible nitric oxide synthase (iNOS), c-Fos, and p50, therefore inhibiting the binding of nuclear factor-*k*B (NF-*k*B) and activator protein-1 (AP-1) with DNA. These transcription factors contribute to cell inflammation and apoptosis. Curcumin prevented lipid peroxidation and oxidative stress. It also restored the balance of the Bcl-2/Bax ratio and inhibited transforming growth factor beta (TGF-*b*), which suppressed liver cell apoptosis (Pramono *et al.*, 2018). Calebin-A lowered the regulation of tumor necrosis factor-alpha (TNF-*a*), which consequently prevented the NF-*k*B activation (Tyagi *et al.*, 2017). Calebin-A inhibited COX, an enzyme that contributed to inflammation and cytochrome P450 1A2 (CYP1A2) and CYP2D6, which were responsible for transforming xenobiotics into pro-carcinogenic compounds that may damage liver cells (Oliveira *et al.*, 2015).

DEN þ JTK had 76.4% lower SGPT and 42.9% lower SGOT activity compared to the normal diet þ DEN group. This emphasized that JTK protected the liver from DEN induction. The hepatoprotective activity of JTK was contributed by xanthorrhizol, curcumin, calebin-A, phenolic compounds and terpenoid compounds (Zubaidah *et al.*, 2023). However, the SGPT and SGOT activity of the DEN þ JTK group was also lower than the DEN þ JTB group. This proved that kombucha fermentation enhanced the hepatoprotective activity of the raw material.

During the fermentation of black tea kombucha, yeasts and bacteria formed glucuronic acid, DSL and released complex polyphenol and flavonoid compounds (Wang *et al.*, 2013; Chakravorty *et al.*, 2016). These compounds were also found in JTK. Glucuronic acid increased the polarity of xenobiotics, decreased their toxicity, facilitated liver absorption, metabolism and excretion of xenobiotics through bile and urine. DSL produced by lactic acid bacteria (LAB) and *Gluconacetobacter sp.* inhibited *b*-glucuronidase, an enzyme responsible for the cleavage of glucuronic acid and toxicant. By this inhibition, the toxicant would bind to glucuronic acid and be easier to be excreted (Wang *et al.*, 2013). Microbes also released phenols that were bound to the substrate's matrices. Kombucha yeasts are dominated by *Candida*, a genus that possesses the ability to degrade complex polyphenol and flavonoid

compounds into simpler compounds (Chakravorty *et al.*, 2016), which increases the number and antioxidant activity of the compounds.

DEN þ black tea kombucha had 49.1% lower SGPT and 14.3% lower SGOT activity compared to normal diet þ DEN. Black tea exhibited good hepatoprotective activity due to its anti-inflammatory and antioxidative activity. Black tea kombucha inhibited liver cell inflammation and apoptosis by maintaining the mitochondrial membrane potential so that the cells did not activate caspase-3, an enzyme that induces cell death (Lee *et al.*, 2019). Black tea kombucha also possessed glucuronic acid and DSL. On the other hand, black tea itself contained various polyphenolic compounds, predominantly catechin. During fermentation, microbes hydrolyzed catechin and converted other polyphenolic compounds into catechin, which increased the antioxidant activity (Jakubczyk *et al.*, 2020).

The SGPT and SGOT activity of DEN þ JTK were lower than the DEN þ black tea kombucha group. Javanese turmeric contains xanthorrhizol, curcumin, phenolic compounds and triterpenoids which do not exist in black tea (Puteri *et al.*, 2020). During kombucha fermentation, microbes released these hepatoprotective compounds and produced organic acids such as glucuronic acids and DSL by using sugars added to the substrate (Martínez-Leal *et al.*, 2018). It was assumed that there was a synergy between hepatoprotective compounds of Javanese turmeric and microbial activity during fermentation.

3.3 Lipid peroxidation level

MDA was a product of a secondary oxidation process that could be found in the cell membranes. Higher MDA levels indicate higher oxidation levels in the body (Castro *et al.*, 2015). Table 2 shows the result of MDA measurements. The normal diet group resulted in a significantly higher MDA level compared to the normal diet þ JTK group. This proved that JTK was able to decrease oxidative stress levels in healthy mice. The normal diet þ DEN group had the highest MDA level. DEN was metabolized in the liver by P450 monooxygenase, resulting in active ethyl radicals (Mansour *et al.*, 2019). These free radicals increased lipid peroxidation, suppressed antioxidant enzyme activity and increased the number of toxic metabolites such as 4-hydroxynonenal and MDA (Zhang *et al.*, 2021).

DEN þ JTB and DEN þ JTK performed a lower MDA level compared to the normal diet þ DEN group. Javanese turmeric protects the liver from DEN-caused oxidative stress by increasing glutathione reductase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase activity (Lukitaningsih *et al.*, 2020). Xanthorrhizol and curcumin

Table 2.
MDA levels in blood serum of mice (n ¼ 7)

Treatments	MDA (nanomol/mL)	%decrease
Normal diet	4.7^{ab} 6 0.25	0.0
Normal diet þ Javanese turmeric kombucha	3.4^{c} 6 0.09	27.1
Normal diet þ DEN	5.1^{a} 6 1.00	0.0^{c}
DEN þ Nonfermented Javanese turmeric beverage	3.8^{bc} 6 0.23	25.6^{b}
DEN þ Javanese turmeric kombucha	2.9^{c} 6 0.08	42.9^{a}
DEN þ black tea kombucha	2.9^{c} 6 0.06	42.4^{a}

Notes: MDA ¼ malonaldehyde; DEN ¼ diethylnitrosamine; MDA decrease percentage of normal diet þ Javanese turmeric kombucha was calculated based on the MDA value of normal diet MDA decrease percentage of DEN þ nonfermented Javanese turmeric, DEN þ Javanese turmeric kombucha, and DEN þ black tea kombucha was calculated was calculated based on the MDA value of normal diet þ DEN; Data is presented in means 6 SD; data followed by different letters shows a significant difference ($p < 0.05$)

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donated hydrogen to free radicals and chelated oxygen to prevent oxidation (Noreen *et al.*, 2017). Xanthorrhizol can prevent LDL peroxidation due to its phenolic hydroxyl group and bisabolene backbone (Oon *et al.*, 2015). Xanthorrhizol chelated $Cu^{2þ}$, decreased the catalyst of LDL peroxidation and improved the activity of superoxide dismutase (SOD) and catalase that resulted in less MDA formation.

The MDA level of DEN þ JTK was less than DEN þ JTB. During kombucha fermentation, microbes released enzymes that freed bound antioxidants. Acetic acid bacteria oxidized sugars into aldehydes and organic acids, which donated $H^{þ}$ to free radicals (Hardiwati *et al.*, 2019). LAB produced curcumin/dehydrocurcumin reductase that reduced curcumin into its analog (THC, hexahydrocurcumin and octahydrocurcumin) in the presence of nicotinamide adenine dinucleotide phosphate (Huang *et al.*, 2018). THC stabilized hydroxyl radicals, reduced MDA and increased SOD and catalase better than curcumin (Morales *et al.*, 2015; Trivedi *et al.*, 2020).

DEN þ black tea kombucha displayed the lowest MDA level. Nonfermented black tea is known to have good antioxidant activities due to its components (catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, theaflavin and thearubigin). Black tea kombucha possessed higher levels of phenolic compounds compared to nonfermented black tea (Jakubczyk *et al.*, 2020). Kombucha also had gluconic acids and thearubigin derivatives that were able to stabilize free radicals. The presence of DSL and ascorbic acids also helped eliminate toxicants which could be metabolized into free radicals inside the body (Morales, 2020). The MDA level of DEN þ black tea kombucha showed no significant difference with DEN þ JTK and normal diet þ JTK.

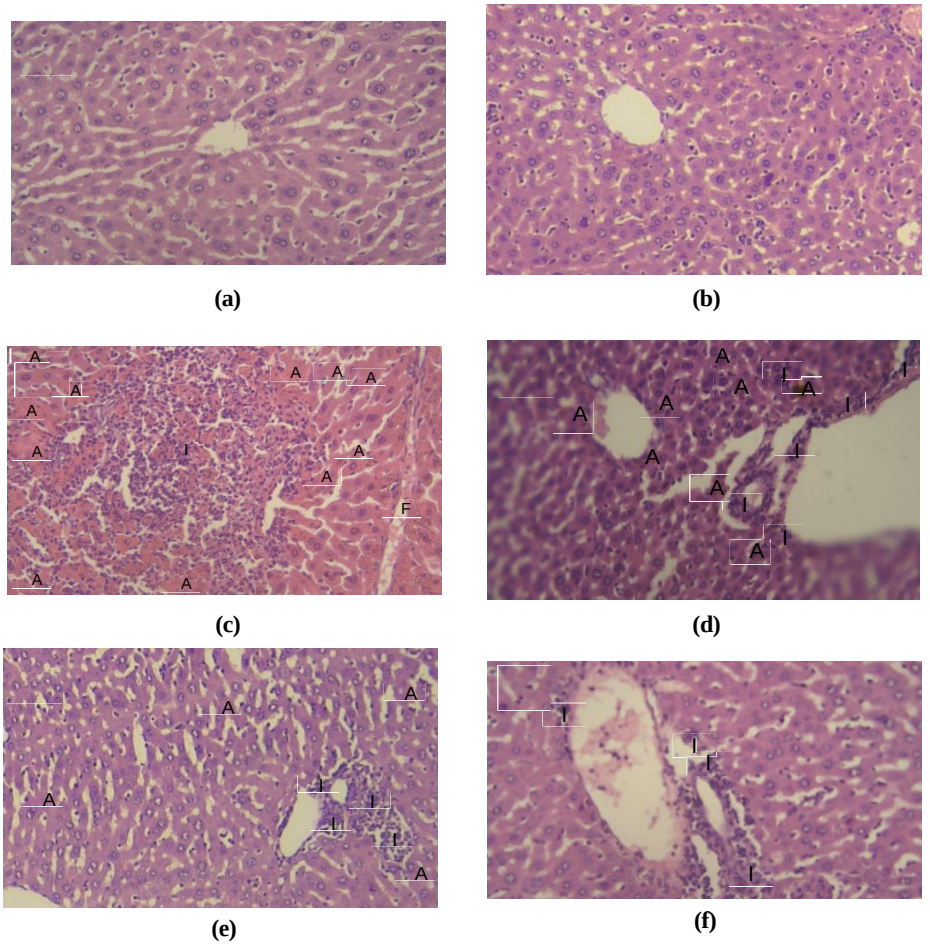
3.4 Liver histology

In Figure 1, normal diet mice liver and normal diet þ JTK mice liver showed purple cytoplasm, dark nucleus, clear cell lining and distinct central vein. This histology was in concordance with the description of normal mice liver histology (Devaraj *et al.*, 2014). DEN þ normal diet showed ambiguous cell linings, bright pink cytoplasm and loss of nuclei. A group of cells were degraded and replaced by inflammatory cells, which was caused by continuous lobule damage and indicated liver hepatitis (Krishna, 2017). The reddish cells with no nuclei were apoptotic bodies, indicating liver cell death by DEN (Boyd *et al.*, 2020).

The DEN þ JTB group showed lesser bile inflammation and apoptotic bodies than the DEN þ normal diet group. The DEN þ black tea kombucha group underwent bile inflammation and had some apoptotic bodies, although the degraded cell area was not as large as the normal diet þ DEN group. DEN þ JTK experienced central vein inflammation, but not as serious as other DEN-induced groups. No apoptotic bodies were seen in the DEN þ JTK group, indicating that the cells were not seriously injured by DEN and had not yet experienced apoptosis.

Figure 2 shows that the DEN þ normal diet group had the highest damaged cell number. DEN could induce DNA methylation which triggers apoptosis (Tolba *et al.*, 2015). DEN induction activated macrophage and neutrophils, which consequently produced proinflammatory cytokine and chemokine. DEN also stimulated the infiltration of inflammatory cells such as lymphocytes, eosinophils and Kupffer cells (Mansour *et al.*, 2019). Liver inflammation can increase vein pressure and decrease intrahepatic nitrite oxide. The suppression of nitrite oxide reduced the ability of vasodilatation and triggered cirrhosis (Vizzutti *et al.*, 2013).

DEN þ JTK and normal diet þ JTK groups had the lowest number of damaged cells, followed by DEN þ JTB and DEN þ black tea kombucha groups. This proved that JTK had better hepatoprotective activity compared to JTB and black tea kombucha. The damaged



Notes: (a) Normal diet; (b) normal diet + JTK; (c) normal diet + DEN; (d) DEN + nonfermented JTB; (e) DEN + black tea kombucha; (f) DEN + JTK; 400× magnification
I = inflammation; A = apoptotic bodies; F = fibrosis
Source: Created by authors

Figure 1.
Mice liver histology

cell number for DEN þ JTK was nearly the same as the normal diet þ JTK group. This indicated that JTK was able to alleviate liver damage and restore the liver to its normal condition.

4. Conclusions

Fermentation enhanced the hepatoprotective activity of Javanese turmeric due to the release of bioactive compounds and new bioactive compound formation. As evidenced by the least damaged liver-cell numbers, JTK demonstrated better hepatoprotective activity compared to JTB.

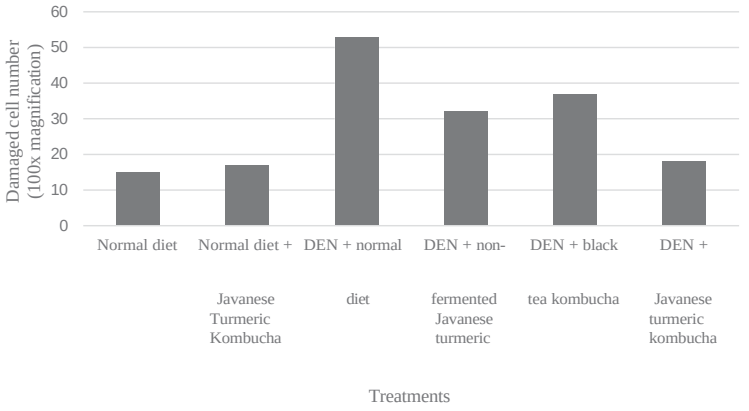


Figure 2.

Damaged cell number on the field of view of 100x magnification

Note: DEN = diethylnitrosamine
Source: Created by authors

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