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The distinctive hepatoprotective activity of turmeric kombucha (*Curcuma longa*) induced by diethylnitrosamine in Balb/C mice

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Abstract

This study aims to investigate the potential hepatoprotective activity of turmeric kombucha before and after fermentation and to compare such distinctive activity in turmeric kombucha versus turmeric essence beverage (turmeric beverage without fermentation). Liquid chromatography-mass spectrometer (LC-MC) analyses revealed the presence of bioactive compounds in turmeric kombucha and turmeric essence beverages. *In vivo* tests appraised the levels of alanine transaminase (ALT), aspartate transaminase (AST), malondialdehyde (MDA) in Balb/C mice and the histology of their livers was determined. Upon successful fermentation process new compounds such as: tetrahydrocurcumin, ferulic acid, glucuronidated curcumin, cyclofenil, acetic acid, glucuronic acid, and D-saccharic acid-1,4-lactone were produced in turmeric kombucha, which were not found in non-turmeric kombucha. The positive effect of fermentation has boosted the hepatoprotective activity of turmeric kombucha through the release of compounds and the production of new bioactive compounds. Therefore, fermented turmeric kombucha had a greater effect on the hepatoprotective activity compared to turmeric essence beverage in Balb/C mice.

Keywords: Turmeric, kombucha, fermentation, hepatoprotective, diethylnitrosamine

1. Introduction

The liver is the main organ that plays a part in the metabolism of drugs and toxic chemicals. Excessive exposure to toxins can cause hepatotoxicity (Maran et al., 2022). Several factors that contribute to liver toxicity include genetic, carcinogenic, and interactions with drugs and alcohol (Malaguarnera et al., 2012). Exposure to chemicals such as diethylnitrosamine (DEN) can induce liver damage and cause oxidative stress, inflammation, and deoxyribonucleic acid (DNA) destruction (Al-Rejaie et al., 2009). Liver damage is triggered when enzymes in the liver undergo lysis and are released into the blood. Compounds that can maintain and repair liver damage are called hepatoprotectives (Maran et al., 2022).

Turmeric is a medicinal plant with functional biological properties and benefits for human health. The bioactive compounds contained in turmeric are curcuminoids, essential oils, tannins, and minerals. It was reported that 2%-5% of turmeric essential oils consisted of phenylpropane turmerone derivatives (aryl-turmerone, alpha turmerone, and beta turmerone) (Goenka et al., 2021). Curcumin has been known to have antioxidant activity, as a radical scavenger, and as a catalyst for the formation of hydroxyl radicals (Bimonte et al., 2013). However, the bioavailability of active compound in curcumin is relatively low due to binding to other compounds.

The fermentation process is one of the food processing methods in which large substrates are broken down into simpler ones assisted by the action of microorganisms. Kombucha is a traditional drink from the fermentation process of sweet tea with a mixed culture of bacteria and yeast. The mixed culture is commonly known as SCOBY (symbiotic culture of bacteria and yeast) which produces a floating biofilm known as microbial cellulose layer or ‘nata’ (Zailani & Adnan, 2022). The substrate often used is steeped tea, so ‘nata’ is also known as "tea mushroom" or tea fungus" (Battikh et al., 2012). Zubaidah et al. (2021) has explored the chemical, microbiological, and antibacterial characteristics of turmeric kombucha, concluding that turmeric can be processed as a kombucha with notable microbiological and antibacterial activity. There have been no research on turmeric kombucha as a hepatoprotective by the time this article was written. This study was conducted to determine the potential hepatoprotective property of turmeric kombucha.

2. Materials and methods

2.1.Materials

Turmeric (*Curcuma longa*) was obtained from a local traditional market in Malang, East Java, Indonesia. Commercial kombucha starter (SCOBY), sugar, and chemicals were obtained from local distributors. SCOBY consists of acetic acid bacteria (AAB) Acetobacteraceae and osmophilic yeast (Filippis et al., 2018). DEN was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ketamine HCl injection (Bernofarm; anesthesia) was obtained from Bioscience Institute Universitas Brawijaya (Malang, East Java, Indonesia). Thirty male Balb/c mice as the experimental animals (6 wks old, 20-30 g). Water and feed were given ad libitum during 1-week period of acclimatization.

2.2.Kombucha turmeric and turmeric essence beverage solution preparation and analysis

Kombucha preparation and analysis was done according to previous research by Zubaidah et al., 2021. Turmeric was peeled and washed, sliced to ± 1 cm thick, dried in a dry cabinet at 70ºC for 12 h, and grinded using a blender (Philips, Amsterdam, Netherlands). Turmeric powder was brewed in hot water with a ratio of 1:10 (5 g of powder in 500 mL of water) for 5 min, 10% of sugar was added, and after cooling, 10% (v/v) of SCOBY starter was added. The mouth of the jar was covered with a cloth and tied. The jar was placed in a room that was not exposed to direct sunlight and at room temperature (30ºC) to ferment for 12 d. Non-fermented turmeric essence beverage was prepared with a concentration of 1.2% (6 g of powder in 500 mL of water) and was run through the same procedures as turmeric kombucha, without the addition of a kombucha starter.

2.3.Identification of the components of turmeric kombucha bioactive compound

LC-MS analysis was carried out using a high-performance liquid chromatography-mass spectrometer (LC-20A, Shimadzu Corporation, Kyoto, Japan) equipped with a Waters 2695 preconditioner pump (Waters Corporation, MA, USA). The MS calibration used was Kromtekindo PRO\ACQUDB/Mass. MS scan was carried out with an initial mass of 50.0/s and final mass of 1200.0, scan time was 5.00, interscan time was 0.10 s, start time was 0.0 min, and end time was 50 min. The storage volume used was 50 L, flow ramp was 0.10, flow was 0.20 mL/min, stop time was 35 min, column temperature was 40ºC, column temperature limit was 10ºC, minimum pressure was 0.0 Bar, maximum pressure was 300 Bar, pre-column volume was 0 L, column type 2, with a size of 1 mm x 100 mm. Solvent ‘A’ was 10% methanol, solvent ‘B’ was 90% water, solvent ‘C’ was 0 formic acid, and solvent ‘D’ was 0 acetonitrile. Draw speed; needle depth was 1/mm, sample temperature was 20ºC, and sample limit temperature was 20ºC.

2.4.Animal experiment and analysis

Testing of hepatoprotective activity was carried out using the *in vivo* method with 30 male Balb/c mice (6 wks old, 20-30 g). The research was approved by Brawijaya University Research Ethics Committee (Ethical Clearance No. 104-KEP-UB-2021). Grouping of the mice was carried out according to the experimental design with 10 treatments (Table 1). Turmeric kombucha and turmeric essence beverage were given daily for 3 wks, with the induction carried out only after then. DEN with a dose of 100 mg/kg was given through an intraperitoneal injection process at the rate of 1 injection/wk for 2 wks. During the DEN injection treatment, turmeric kombucha and turmeric essence beverage were still being given, with an incubation period of 1 wk. Mice without DEN injections were treated according to the grouping. On the 49th day, surgery was performed after fasting for 24 h from the last day of treatment. An anesthesia process was used during the induction of 0.2 mL ketamine (50 mg/kg). During surgery, blood serum samples were taken from the heart and liver. Parameters observed were alanine transaminase (ALT) activity, aspartate transaminase (AST), malondialdehyde (MDA), and liver histology (Fig. 1).

2.4.1. ALT and AST enzyme *(*Modification Devaraj et al., 2014)

The clotted blood samples were centrifuged at 3000 rpm (3461 x g in a EBA 200, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at room temperature (30^{o}C) for 15 min to separate the cell nucleus from the blood serum. Blood serum was then taken and biochemically tested for the amount of AST and ALT enzymes.

2.4.2. MDA enzyme (Modification Devaraj et al., 2014)

As much as 10% of the liver homogenate was mixed into 0.1 M Tris-HCl buffer pH 7.4 at 40^{o}C. The sample was homogenized (VELP Scientifica Srl, Usmate, Italy) with at 1000 rpm for 2 min. The homogenate was centrifuged at 1000 rpm at 40^{o}C for 10 min to separate the nucleus and cell solids. The supernatant was tested for the amount of MDA to see the level of liver oxidation.

2.4.3. Histopathological observation (Modification Jantararussamee et al., 2020)

Histopathological observations of mice were carried out by taking some liver samples from each group by dissection. The results of the dissection were dehydrated with 50%-100% ethanol and given paraffin with a thickness of 5 cm. The paraffin-treated sections were then stained with hematoxylin and eosin (HE) to color the cell parts and observed under a light microscope (Olympus Corporation, Tokyo, Japan) with magnifications of 40x, 100x, and 400x.

2.5. Statistical analysis

The statistical analysis was carried out by comparison of all the groups. Analysis of variance (ANOVA) was used and followed by Fisher's exact test at $p<0.05$. All statistical analyses were carried out using Minitab software (17.0 version, Minitab, LLC).

3. Result and discussion

3.1.Turmeric kombucha and turmeric essence beverage characteristics

Turmeric kombucha and turmeric essence beverage were used as treatments to mice. Physicochemical and microbiological analysis were conducted prior to the *in vivo* procedures. The higher the concentration of turmeric, then the lower the microbe total and acid total obtained. The higher the total phenol concentration of turmeric, then the higher the antioxidant activity. The best treatment results were obtained with 1% of turmeric kombucha concentration (Zubaidah et al., 2021).

The characteristics of turmeric kombucha and turmeric essence beverage found by Zubaidah et al. (2021) are shown on Table 2. Turmeric kombucha showed higher total phenolic content and antioxidant activity elevation compared to turmeric essence. Turmeric kombucha also had a higher total of titratable acid, lower pH, and an increase of the AAB total. This was due to the addition of kombucha starter. Kombucha starter mainly comprised of bacteria and yeast, which led to them influencing the microbial characteristics of turmeric and black tea kombucha. According to Zubaidah et al. (2021), black tea kombucha recorded 1.3×10^{8} CFU/mL of total microbes on day-14, higher than the turmeric kombucha with 2.0×10^{7} CFU/mL. Microbial activity results in the

breakdown of turmeric bioactive compounds. Turmeric kombucha showed and increase of total titratable acid, decrease of pH, higher total phenolic content, and lower IC50 value which enabled better free radical degradation than turmeric essence beverage. This was due to the existence of organic acids produced by microorganisms during fermentation. This proved that kombucha fermentation increased total phenolic content and antioxidant activity of turmeric.

3.2.Components of bioactive compounds in turmeric kombucha and turmeric essence beverage

Identification of chemical compounds contained in turmeric kombucha and turmeric essence beverage using LC-MS (Table 3) revealed that they contained phenolic compounds, curcumin, demethoxycurcumin, bisdemethoxycurcumin, several compounds derived from curcumin, and organic acids. Chemical compounds detected in the phenolic group were nitrophenol, phenol, and quinoline. Phenol compounds are secondary metabolites of plant metabolism that attach to metal ions which can fight free radicals and increase antimicrobial activity (Cavalcanti et al., 2012). Phenolic compounds have functional abilities such as cardiovascular inhibition, anticancer, and chronic disease prevention (Soto-Quintero et al., 2019).

Chemical compounds detected in the curcuminoids group were curcumin, bisdemethoxycurcumin, and demethoxycurcumin. The derivative components of the curcumin compounds consisted of ferulic acid, acetylsalicylic acid, guaiacol, eugenol, licopyranocoumarin, and phenyl. Ferulic acid is an acid consisting of trans-cinnamic acid which has methoxy and substitution of hydroxyl on the phenyl ring. Ferulic acid has bioactivities as an antioxidant, anti-inflammatory, inhibitor of apoptosis, and cardioprotective prevention. Ferulic acid is a chemical compound that is commonly found in plants, belonging to a group of secondary metabolites that bind to esters, glycosides, components of lignin, and tannins (Mattila & Kumpulainen, 2002). Based on the chemical structure, it can be divided into benzoic acid derivatives by substitution of hydroxyl and methoxy groups and phenolic acids. Ferulic acids such as caffeic, p-coumaric, sinapic acid, and vanillin acid are cinnamic acid derivatives (Bezerra et al., 2017).

Acetylsalicylic acid is a chemical compound that functions as an analgesic drug or pain reliever. Acetylsalicylic acid can bind and acetylate serine residues in cyclooxygenase (COX), resulting in decreased prostaglandin synthesis, platelet aggregation, and inflammation. Acetylsalicylic acid has analgesic, antipyretic, and anticoagulant properties. Research conducted by Purpura et al. (2018) reported that curcumin significantly reduced pain in the legs of experimental rats. Prostaglandins are known to reduce pain receptors through the COX and lipoxygenase (LOX) pathways. Conditions like this can suppress COX-2 and 5-LOX which are enzymes that cause pain. Curcumin showed a significant antipyretic effect with decreasing rectal temperature. The decrease in temperature can be caused by the presence of acetylsalicylic acid which can inhibit prostaglandins (Hatcher et al., 2008).

3.3. Hepatoprotective activity

3.3.1. Alanine transaminase

ALT is an enzyme present in the cytosol of liver parenchyma cells and thus is a more specific parameter to analyze liver damage. If there was damage to the liver, the cell would undergo lysis and ALT enzymes would come out of the cells and be carried in the blood circulation. This indicated that the ALT enzyme was detected in the analysis of blood serum *(*Jilkova et al., 2019). Treatment with DEN can affect the activity of ALT in the blood serum of mice. The blood serum of the positive control group (normal diet + DEN) showed higher values than the negative control group (normal diet). ALT activity decreased after the administration of turmeric essence beverage and turmeric kombucha of various concentrations. The administration of turmeric kombucha with a concentration of 0.5 mL/20 g BW showed the largest decrease among the DEN-induced groups, which was 20.851 U/L (Table 3). The normal diet group with DEN induction had the highest ALT value, where there was an increase in the ALT value to 41.147 U/L. DEN damages liver cells, causing lysis and triggering liver cell death. DEN can be metabolized in dysenterylabular hepatocytes followed by oxidative DNA damage reactions (Jilkova et al., 2019). After DEN induction and administration of turmeric essence beverage at a dose of 0.5 mL/20 g BW, the ALT value was reduced to a value of 30.451 U/L. The treatment with turmeric kombucha had lower ALT activity than the turmeric essence beverage treatment. Turmeric kombucha with various concentrations had a higher ability to reduce ALT activity in mouse blood serum. The difference in dosages of turmeric kombucha and turmeric essence beverage showed a significant difference in decreasing ALT activity ($p<0.05$). The reduction of the ALT enzyme in blood serum was because the ability of curcumin to fight free radicals and induce arachidonic acid metabolism through the COX and LOX pathways (Ak & Gülçin, 2008). The results of research conducted by Bimonte et al. (2013) showed that curcumin can prevent liver toxicity and reduce ALT levels caused by methotrexate induction.

3.3.2.Aspartate transaminase

AST is an enzyme found in the cytosol and mitochondria of liver cells, cardiac muscle cells, striated muscles, and kidneys (Jilkova et al., 2019). This indicates that high AST values are not only caused by damage to liver cells but can also occur due to the presence of AST in other cells. If liver cells are lysed, the enzyme will be carried out in the blood circulation so that it can be detected in blood serum analysis (Castro et al., 2015). Treatment with DEN can affect AST activity in the blood serum of mice. The blood serum of the positive control group (normal diet + DEN) shows a higher value than the negative control group (normal diet). AST activity decreased after the administration of turmeric essence beverage and turmeric kombucha of various concentrations. The administration of turmeric kombucha resulted in a higher reduction activity than turmeric essence beverage (Table 4). The increase in the value of AST activity in the positive control group was 40.739 U/L. After the administration of turmeric kombucha and turmeric essence beverage of various concentrations, there was a decrease in AST activity in the blood serum of mice. Notwithstanding, the decrease in AST value in turmeric essence beverage was within normal limits with the lowest value at a dose of 0.5 mL/20 g BW which was 30.077 U/L,

while in turmeric kombucha the lowest value was 20.110 U/L within normal limits. This indicates that the higher the dose given, the lower the value of AST activity in the blood serum of mice.

The normal AST value for mice is 8-40 U/L. Curcumin is a compound found in turmeric with functions as a hepatoprotective, such as antioxidant activity, anti-inflammatory, antimicrobial, and anticarcinogenic (Karimian et al., 2017). Curcumin and curcumin derivatives such as 5-benzo [1,3] dioxol-5-il-1-phenyl-penta-2,4-dien-1 have the ability as hepatoprotectives to protect and repair damaged liver cells. According to research conducted by Kapelle et al. (2020), the increase in turmeric hepatoprotective activity was due to microbial activity during the kombucha fermentation process. According to Acosta-Cota et al. (2019), yeast and *Gluconacetobacter* sp. formed glucuronic acid during the fermentation of kombucha. Identification with LC-MS of turmeric kombucha found compounds of organic acids which were glucuronic acid and 1,4-lactone D-saccharic acid (DSL). Glucuronic acid can bind to toxic metabolites or compounds that will be eliminated from the body so these compounds are more water soluble and their toxic activity is reduced. DSL in kombucha tea is a hepatoprotective detoxifier and can curatively maintain liver pathophysiology. In addition to glucuronic acid, it has the potential to clear hepatotoxins caused by toxins such as acetaminophen, carbon tetrachloride, hydrocarbon carcinogens, nitrosamines, and aromatic amines (Bhattacharya et al., 2011).

3.3.3. Lipid peroxidation level

MDA is a product of fat oxidation. The high the levels of MDA the high the levels of fat oxidation in the body. Lipid peroxidation has a role in the pathogenesis of tissue injury, especially in damage caused by several toxic substances (Dzoyem et al., 2014). Normal diet + DEN induction treatment showed the highest MDA levels. Turmeric kombucha and turmeric essence beverage decreased MDA levels. The lowest MDA levels were found in 0.5 mL/20 g BW turmeric kombucha induced mice (Table 4). This was due to turmeric kombucha containing more bioactive compounds, organic acids, and microorganisms compared to turmeric essence beverage. There were several bioactive compounds derived from curcuminoids that have the functional properties of preventing liver damage. In addition, several organic acid compounds in turmeric kombucha could prevent liver damage such as glucuronic acid and DSL, these compounds were not found in turmeric essence beverage. The higher the dose of kombucha, the lower the MDA levels in the mice's serum. This increase in antioxidant activity reduced lipid peroxidation and prevented the formation of MDA (Sobhani et al., 2020). Organic acids such as acetic acid and glucuronic acid have high antioxidant activity. Kombucha was able to reduce liver damage caused by oxidative stress (Gharib, 2009). Glucuronic acid is a bioactive compound in kombucha with high antioxidant activity as a detoxifier in the liver through the glucuronidation process. Glucuronidation is a xenobiotic conjugation process such as; acetylaminofluorene (carcinogenic), aniline, benzoic acid, and steroid compounds. The conjugation process with glucuronyl transferase enzyme is catalyzed by UDP-glucanosyltransferase (Alvarenga et al., 2018; Coton et al., 2017).

3.4. Liver Histology

Liver histology was performed to determine the condition of the cells in the liver, observations were made using preparations from the liver. Liver damage is characterized by the occurrence of inflammatory cell damage, fibrosis, and the formation of acidophilic bodies/apoptotic bodies. The negative control group/normal diet (Fig. 2a) shows that the liver cells looked normal, where the condition of the cells stained with HE had purple cytoplasm, the cell nucleus was clear and had a dark purplish color, the boundaries between the cells were visible, and the central blood vessels were visible. Normal histology has a brownish-red color, shiny, sharp edges, a smooth texture, good cytoplasmic conditions, a prominent nucleus, and sinusoidal spaces. It also has liver lobules and a uniform pattern around polyhedral hepatocytes from the central vein to the periphery (Jantararussamee et al., 2021; Jeyadevi et al., 2019; Mondal et al., 2019). Normal diet groups fed with turmeric essence beverage and turmeric kombucha display similar liver histology to the negative control group (Fig. 2c and 2g). The positive control group (normal diet + DEN) shows the histology of a damaged liver due to the toxicity of DEN (Fig. 2b). Cells had a light pink color and some cells did not have a cell nucleus. The boundaries between liver cells were not clearly visible. Liver cells underwent degradation and inflammation occurred in some cells. Induction of DEN can cause hydropic degradation, mitosis, pseudo-nucleus, apoptosis, and liver necrosis (Santos et al., 2017). The treatment of turmeric kombucha and turmeric essence beverage showed changes in liver histology for the better. The 0.1 mL/20 g BW dose from turmeric essence beverage and kombucha improved cell boundaries and nucleus prominence (Fig. 2d and 2h), then doses of 0.3 mL/20 g BW and 0.5 mL/20 g BW produced almost normal liver histology (Fig. 2e, 2f, 2i, and 2j).

3.5.Total cell damage

Turmeric is a rhizome that contains curcumin as an anti-inflammatory bioactive. Administration of turmeric kombucha and turmeric essence beverage can reduce and prevent inflammation. Curcumin can inhibit proliferation and reduce inflammation. In addition, it can also reduce levels of MDA, glutathione, nitric oxide (NO), and tumor necrosis factor (TNF) and increase catalase, superoxide dismutase (SOD), and glutathione transferase (GST) activity in the liver (Tokaç et al., 2013). Based on the histology data, the results show liver cell damage due to DEN induction through several damaged and dead cells (Table 5).

4. Conclusion

This study shows that the fermentation process can produce other compounds in turmeric kombucha that are not detected in turmeric essence beverage. Fermentation affects the hepatoprotective activity of turmeric through the release of compounds and the production of new bioactive compounds. Therefore, fermented turmeric kombucha offers greater effect on the hepatoprotective activity compared to turmeric essence beverage in experimental animal.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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496 **Tables**

497

498 **Table 1**. List of formulations to test the hepatoprotective property of turmeric kombucha and
499 turmeric essence beverages

500

Treatment Group	Description
Control negative (P0)	Normal mice by feeding healthy mice/mice
Control positive (P1)	Mice Normal diet + DEN
P2	Mice non-DEN+ Turmeric essence beverage dose 0.3 mL/20 g BW
P3	Mice DEN + Turmeric essence beverage dose 0.1 mL/20 g BW
P4	Mice DEN + Turmeric essence beverage dose 0.3 mL/20 g BW
P5	Mice DEN + Turmeric essence beverage dose 0.5 mL/20 g BW
P6	Mice non-DEN + Turmeric kombucha dose 0.3 mL/20 g BW
P7	Mice DEN + Turmeric kombucha dose 0.1 mL/20 g BW
P8	Mice DEN + Turmeric kombucha dose 0.3 mL/20 g BW
P9	Mice DEN + Turmeric kombucha dose 0.5 mL/20 g BW

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503

504 **Table 2.** Turmeric kombucha and turmeric essence beverage characteristics (Zubaidah et al.,
505 2021)

Parameter	Turmeric kombucha	Turmeric essence beverage
pH	3.8	7.4
Total titratable acid	1.24%	Not detected
Total phenolic content	137.28 mg GAE/mL	94.25 mg GAE/mL
IC_{50} antioxidant activity	76.16 ppm	106.59 ppm
Total microbial cells	2.70×10^7 CFU/mL	Not detected

506

507 **Table 3**. Identified chemical compounds in turmeric kombucha and turmeric essence beverage
508 using LC-MS

Component	MW (g/mol)	Retention time	Turmeric kombucha	Turmeric essence beverage	Benefit
Tetrahydrocurcumin(1,7-bis(4-hydroxy-3-methoxyphenyl)	372.4	2.563	√	N/D	Anti-inflammation, Anti-cancer and anti-bacterial
Ferulic acid (4-Hydroxy-3-methoxy cinnamic acid)	194.18	1.277	√	√	Antioxidant, anti-inflammation, apoptosis, and cardioprotective (Bezerra et al., 2017; Mattila & Kumpulainen, 2002)
Acetylsalicylic acid (2-acetyl benzoic acid)	180.31	2.563	√	N/D	Analgesic (Ashok & Upadhyaya, 2013)
Methoxyphenol	124.14	1.772	√	√	Anti-carcinogenic and antioxidant (Sun et al., 2015).
Eugenol	127.39	2.735	√	√	Anti-bacterial, antioxidant, and analgesic
Bisdemethoxycurcumin	308.3	6.127	√	√	Anti-inflammation and lower expression NF-Kβ *(*Ramezani *et al*., 2018).
Demethoxycurcumin	308.3	17.871	√	√	Anti-inflammation and anti-neoplastic (Hoehle et al., 2006)
Curcumin glucuronide	544.5	3.182	√	N/D	Immunosuppressive, antioxidant, anti-neoplastic, cytotoxic, anti-cancer, and anti-tumor (Ming et al., 2020)
Cyclofenil	364.4	1.565	√	√	Ovulation induction, infertility anti-virus (Sayed & El-Kordy, 2014), and inhibition of MCF cell proliferation in breast cancer (Mughal, 2019)
Acetic acid	60.05	14.183	√	N/D	Antioxidant, anti-microbial, toxicity (Jakubczyk et al., 2020; Jayabalan et al., 2014; Tahri et al., 2016), and anti-

					inflammation (Bimonte et al., 2015)
D-saccharic acid-1,4-lactone (DSL)	192.12	18.721	√	N/D	Antioxidant, anti-inflammation, and heart damage (Ireson et al., 2002), anti-diabetes, cytotoxic, hepatoxic, and hepatoprotective (Wang et al., 2014)
Glucuronic acid	397.17	17.234	√	N/D	Antioxidant, hepatoprotective, and anti-inflammation (Martínez-Leal et al., 2020)
Carboxylic acid	477.4	3.182	√	N/D	Prevent liver damage (Rocha-Ramírez et al., 2017) and immunomodulator (Bauer-Petrovska & Petrushevska-Tozi, 2000)
Chloroacetyl-dl-phenylalanine	241.67	1.600	N/D	√	Bacterial xenobiotic metabolites (Aggarwal et al., 2013; Zhao et al., 2018)
Phenyl	364.4	2.356	√	√	Anti-microbial (Pereira et al., 2009), cardiovascular, and anti-cancer (Sim et al., 2019)
Pyrazine	979.0	2.735	√	√	Analgesic, anti-inflammation, antioxidant, anti-cancer, and anti-microbial
Quinazoline	1033.2	2.941	√	√	Anti-inflammation, anti-cancer, anti-inflammation, and anti-microbial (Bimonte et al., 2015; Hatcher et al., 2008)

512 **Table 4**. Hepatoprotective activity of turmeric kombucha and turmeric essence beverage

Treatment group	ALT (U/L)	AST (U/L)	MDA (nanomole/mL)
Normal diet	$25.770^{d} \pm 1.0$	$20.199^{e} \pm 0,8$	$4.319^{ab} \pm 0,7$
Normal diet + DEN	$41.147^{a} \pm 0.4$	$40.739^{a} \pm 1,1$	$5.292^{a} \pm 0,8$
Normal diet + turmeric essence beverage	$20.445^{e} \pm 1.2$	$21.347^{d} \pm 1,3$	$3.812^{b} \pm 0,3$
DEN + dose 0.1 mL/20 g BW	$32.510^{b} \pm 0.4$	$31.958^{b} \pm 3,7$	$4.322^{ab} \pm 0,1$
DEN +dose 0.3 mL/20 g BW	$31.355^{bc} \pm 0.4$	$31.107^{bc} \pm 0,9$	$4.076^{ab} \pm 0,6$
DEN + dose 0.5 mL/20 g BW	$30.451^{c} \pm 1.1$	$30.077^{c} \pm 3,3$	$4.032^{ab} \pm 0,7$
Normal diet + turmeric kombucha	$20.884^{e} \pm 0.8$	$20.454^{de} \pm 0,5$	$3.858^{ab} \pm 0,3$
DEN + dose 0.1 mL/20 g BW	$21.962^{e} \pm 0.1$	$21.738^{d} \pm 0,5$	$4.079^{ab} \pm 0,2$
DEN + dose 0.3 mL/20 g BW	$21.040^{e} \pm 0.8$	$21.341^{de} \pm 1,2$	$3.807^{b} \pm 0,4$
DEN + dose 0.5 mL/20 g BW	$20.851^{e} \pm 0.8$	$20.110^{e} \pm 0,3$	$3.761^{b} \pm 0,1$

513 Note: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MDA: Malonaldehyde;
514 different notations show a real difference (α=0.05); data obtained from the average of 3
515 replications ± SD
516
517

518 **Table 5**. Total cell damage

Treatment	Total of dead cells
Normal diet	$17^{e} \pm 0.8$
Normal diet + DEN	$49^{a} \pm 0.8$
Normal diet + Turmeric Essence Beverage	$16^{e} \pm 1.2$
DEN + dose 0.1 mL/20 g BW	$32^{b} \pm 1.6$
DEN + dose 0.3 mL/20 g BW	$30^{bc} \pm 0.8$
DEN + dose 0.5 mL/20 g BW	$28^{cd} \pm 0.5$
Normal diet + Turmeric kombucha	$15^{e} \pm 0.5$
DEN + dose 0.1 mL/20 g BW	$30^{b} \pm 0.9$
DEN + dose 0.3 mL/20 g BW	$27^{d} \pm 1.2$
DEN + dose 0.5 mL/20 g BW	$26^{d} \pm 0.5$

519 Note: Different notations show a real difference (α=0.05); data obtained from the average of three
520 replications ±SD
521

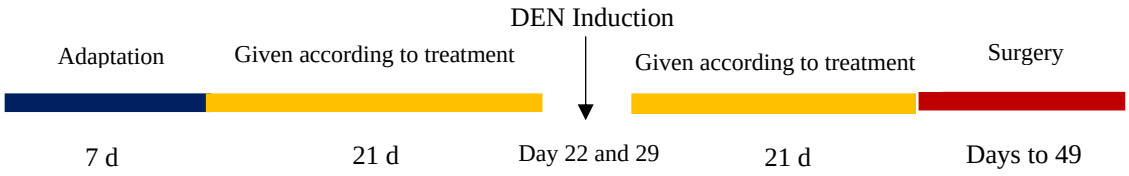
522 **Figure legend**
523
524 **Fig. 1**. Experimental animal treatment procedures, modified (Zheng et al., 2018)
525
526 **Figure 2**. Mice liver histology
527 (a) Normal diet; (b) Normal diet + DEN; (c) Normal diet + turmeric essence beverage; (d) DEN +
528 0.1 mL/g turmeric essence beverage; (e) DEN + 0.3 mL/g turmeric essence beverage; (f) DEN +
529 0.5 mL/g turmeric essence beverage; (g) Normal diet + turmeric kombucha; (h) DEN + 0.1 mL/g
530 turmeric kombucha; (i) DEN + 0.3 mL/g turmeric kombucha; (j) DEN + 0.5 mL/g turmeric
531 kombucha; magnification 400x
532 I: inflammation; A: apoptotic body; F: fibrosis; CV: central vein
533
534

535 **Figures (colors should be used)**

536

537 **Fig. 1.**

538



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541 **Fig. 2.**

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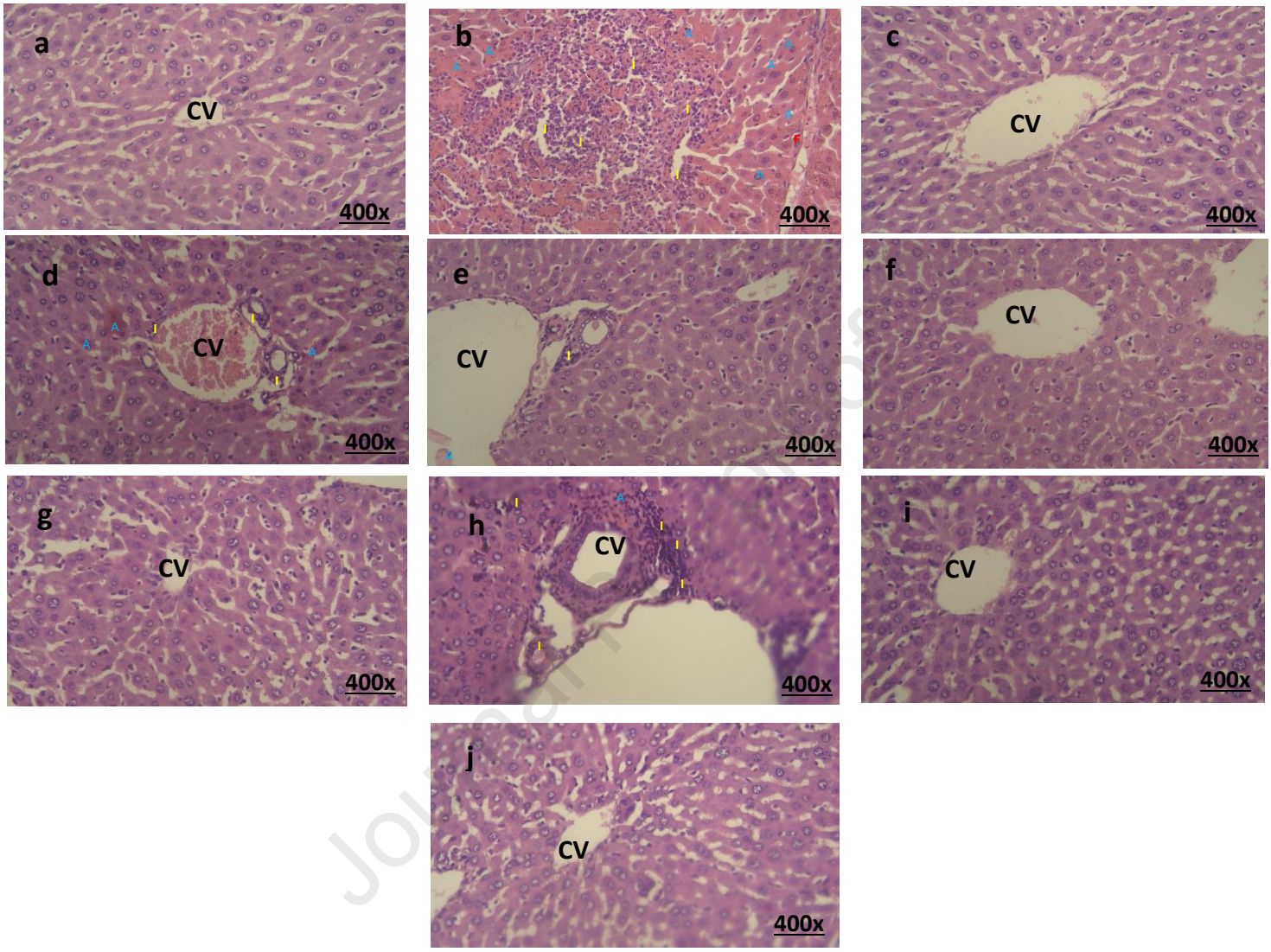
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Author statement

We hereby declare that all the authors of "The distinctive hepatoprotective activity of turmeric kombucha (*Curcuma longa*) induced by diethylnitrosamine in Balb/C mice" have approved the newly revised manuscript to be re-submitted to Food Bioscience. There are no conflicts of interests.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: