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Hepatoprotective Study of Indonesian Water Kefir Against CCl4-Induced Liver Injury in Rats

Running title: Hepatoprotective study of water kefir

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Contribution	**Widhya Aligita**	**Marlia Singgih**	**Entris Sutrisno**	**I Ketut Adnyana**
Concepts or Ideas	X	X	X	X
Design	X	X	X	X
Definition of intellectual content	X	X	X	X
Literature search	X			X
Clinical trial				
Experimental studies	X			X
Data acquisition	X			X
Data analysis	X			X
Statistical analysis	X			X
Manuscript preparation	X	X	X	X
Manuscript editing	X	X	X	X
Manuscript review	X	X	X	X

ABSTRACT

Context: Water kefir is a fermented beverage that is typically made in the home by inoculating a sugar-rich solution with a microbial community (water kefir grains). Several studies on the metabolite content and hepatoprotective effects of water kefir have been published, but CCl4-induced acute liver injury has not been studied.

Objectives: ~~The purpose of this study was~~ to evaluate the efficacy of water kefir in vivo against hepatoprotective CCl4-induced acute liver injury and to in silico investigate metabolites that play an important role in hepatoprotective mechanisms.

Methods: Water kefir significantly and dose-dependently alleviated acute liver injury caused by carbon tetrachloride (CCl4). Water kefir administration at all doses produced results comparable to the positive control (curcuma extract). Furthermore, using molecular docking, the metabolites found in water kefir were evaluated for their role in the NF-κB and Nrf2 signaling pathways.

Results: Molecular docking simulations showed that the 25 metabolites tended to interact with the NF-κB receptor compared to Nrf2. Fumaric acid was the strong metabolite that interacts with the NF-κB receptor with a free energy of binding and inhibition constant of -6.66 kcal/mol and 13.22 μM, respectively.

Conclusions: This study concludes that water kefir has hepatoprotective properties by decreasing inflammation and fibrosis levels.

Keywords: Hepatoprotective; Molecular docking; Metabolites; NF-κB; Nrf2; Water kefir

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INTRODUCTION

In most cases, water kefir is created by blending water kefir grains, sugar, and dried fruit in a container. Water kefir's exact origins are unknown; however, two hypotheses have been proposed regarding its history: the first suggests that water kefir grains were brought to Europe from the Caucasus by soldiers returning from the Crimean War in the late nineteenth century (1); the second theory proposes that water kefir grains originated in Mexico from the *Opuntia cactus* through natural processes (2). Sugary kefir grains, Balm of Gilead, African bees, Japanese beer seeds, Ale nuts, and California bees are some other names for water kefir. Tibi grains and ginger beer plant are other names for water kefir (2–4). Water kefir is appealing to both consumers and researchers due to the variety of microbiota it contains, the fact that it is an alternative to dairy products, the versatility with which it can be flavored, the fact that it is low in calories and sugar, the ease with which it can be produced, and the health benefits it offers.

Water kefir has been used medicinally for a very long time, and recent research has indicated that it may have a variety of positive effects on people's health. It has been demonstrated that water kefir contains non-pathogenic bacteria, in conjunction with the production of organic acids, can inhibit the growth of pathogenic microbes, such as *Shigella sp.*, *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; and also, filamentous fungi such as *Aspergillus ochraceus, A. niger, A. flavus, Penicillium sp.*, and *Rhizopus sp*. (add bibliography(ies)) In addition to its antibacterial properties, water kefir possesses a broad spectrum of pharmacological effects. Some of these therapeutic effects are anti-inflammatory (5,6), antioxidant (6–8), hepatoprotective (9,10), antihyperglycemic and antihyperlipidemic (11,12), anti-edematous (13), antitumor (14), antihypertensive (15), immunomodulant (16), and anti-ulcerogenic (17). However, no studies have been reported on the hepatoprotective effects of water kefir against carbon tetrachloride (CCl_4)-induced liver injury.

Studies have shown that acute liver injury is frequently accompanied by high levels of oxidative stress and inflammatory responses (18). These findings have been found in several studies. The most important signaling pathways that are involved in the regulation of inflammation and antioxidation are the nuclear factor (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and inhibiting NF-κB can reduce the amount of damage done to the liver. For instance, curcumin protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1)) (19). In

addition, methoxy eugenol, a molecule that is derived from nutmeg and Brazilian red propolis, has been shown to exhibit hepatoprotective activity both *in vitro* and *in vivo*. This may be attributed to the fact that it targets the NF-κB signaling pathway, which has been shown to have anti-inflammatory effects (20).

Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage. Additionally, a variety of aromatic and volatile compounds are produced, including ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others (compared to their threshold levels) (21). The chemical constituents of both phytochemicals and secondary metabolites in natural products, including water kefir, are certainly capable of providing various pharmacological effects for the body (22,23). However, an *in silico* study to evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity to speed up the process of identifying and optimizing lead compounds, the *in silico* method has become the front-runner in the race to improve the speed and accuracy of the process of discovering new drugs. This is because the *in silico* method can identify and optimize lead compounds more quickly. Techniques such as molecular docking and molecular dynamics (MD) were able to directly indicate a small number of compounds that have high affinity and selectivity by analyzing how the ligand and target interact with one another (24). Please, the style for indicating bibliographic citations does not comply with JPPRes requirements, please address this and improve it.

There are numerous studies demonstrating the hepatoprotective activity of kefir. Therefore, the authors should state here the novel effect they intend to find, which has not been reported in the scientific literature. Please better state the research problem and how you intend to solve it.

Therefore, the purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl_4-induced rats while also investigating the stability interactions of its metabolites within the NF-κB and Nrf2 receptors using molecular docking study. Our findings suggest that water kefir and its ~~the~~ metabolites it produces could be a promising therapeutic agent for the management of liver injury that is both safe and effective. Please move this last sentence to the Conclusions, it is not valid for an Introduction.

MATERIAL AND METHODS

Materials and Reagents

Carbon tetrachloride, rats, diagnostic kits for alanine aminotransferase (ALT) (Proline, IFCC mod.), aspartate aminotransferase (AST) (Proline, IFCC mod.), Elisa Kit TNF-α (Bioassay Technology Laboratory), Elisa Kit TGF-β (Bioassay Technology Laboratory). Other chemicals used in this study were of analytical reagent grade.

Experimental sample and reference extract

It is necessary to indicate here where these materials were obtained from. Indicate who identified the material. The manuscript should include references to voucher specimens of the plants (deposited in a major regional herbarium) or to the material examined, including their registration number(s). It should be mentioned which parts of the plant have been used. The GPS coordinates of the collection site of the species should also be indicated. It is mandatory that the authors indicate how both the kefir and the turmeric extract were prepared, the quality of these products, etc.

Animals and Experimental Design

Rats (Wistar strain, male, 200-250 g) were maintained on normal pellet food and tap water (*ad libitum?*). Four mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animals Care (NIH publication no. 85-23, revised 1985). In order to develop an animal model with liver injury, the rats received CCl_4 (20% in olive oil) 1.25 mL/kg BW every 2 days via a gavage tube (25). The rats were randomized into five groups after the development of animals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group; with addition (6) negative control group. Each group received group-specific treatment for two weeks, along with administration of 1.25 mL/kg BW of CCl_4 (20% in olive oil) every three days. Serum ALT level, as the main parameter, was measured prior to induction, following induction, and following treatment. Meanwhile, following therapy, serum AST, TNF-α, TGF-β levels, and liver histopathological were evaluated. Please indicate how the serum was extracted in the rats? from which part of the animal and how the blood was extracted. How were AST, TNF-α, TGF-a levels measured? give details of this. How was the liver extracted from the animals? how was the liver histopathological analysis performed.

Only manuscripts of experiments conducted in accordance with the appropriate guidelines will be eligible for publication. When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations **and to approval by the local ethical committee**. **The approval number and the corresponding date must be provided**. It must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

If the Curcuma (what curcuma species, scientific name, please?) extract was used as a reference, the authors should justify here with bibliographic citation where this extract was tested, at this declared dose, as hepatoprotective.

Molecular docking simulation

The PyRx software (26) was used to perform molecular docking experiments to anticipate the manner of binding that happens between metabolites as small-molecule ligands and biological macromolecules. The NCBI PubChem database (https://pubchem.nlm.nih.gov/, accessed on 03 May 2023) was used to derive the three-dimensional structure of water kefir metabolites (27). Target proteins like NF-κB (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/, accessed on 03 May 2023). PyRx program was used to reduce protein, ligand converted to PDBQT (28), then maximized GRID parameter (29) performed docking study (30). The BIOVIA Discovery Studio 2017 R2 program was used to view the protein and ligand complex and distance **(31)**. The BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

Statistical Analysis

All of the information is displayed in the form of individual data points as well as the mean along with the standard error of the mean. The statistical analysis was carried out with the help of Minitab software (version 19.0), and to make comparisons between several different groups, a one-way analysis of variance with Dunnett's post hoc test was utilized. All statistical

graphs were created with Microsoft Excel 2019 in their respective versions. The levels of significance that were considered to have been reached were * $p < 0.05$.

RESULTS

In vivo evaluation of hepatoprotective activity

The serum ALT levels, as the main parameter for the liver damage, were measured prior to and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment AST, TNF-α, and TGF-β levels were also evaluated. These findings were analyzed using a one-way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant rise in blood ALT levels demonstrated the establishment of an animal model with liver injury, according to statistical analysis, following the administration of CCl_4. When compared to the positive control group, ALT serum levels reduced significantly after two weeks of therapy with curcuma extract or water kefir. The three doses of water kefir groups demonstrated equivalent activity when curcuma extract was used as the standard treatment and there was no significant difference between the three doses of water kefir. When compared to the positive control group, AST levels were also reduced dramatically following treatment with curcuma extract or water kefir. TNF-α levels in the water kefir group were significantly lower than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though there was no statistically significant difference in TGF-β levels, the group that received the treatment demonstrated a decrease in TGF-β levels.

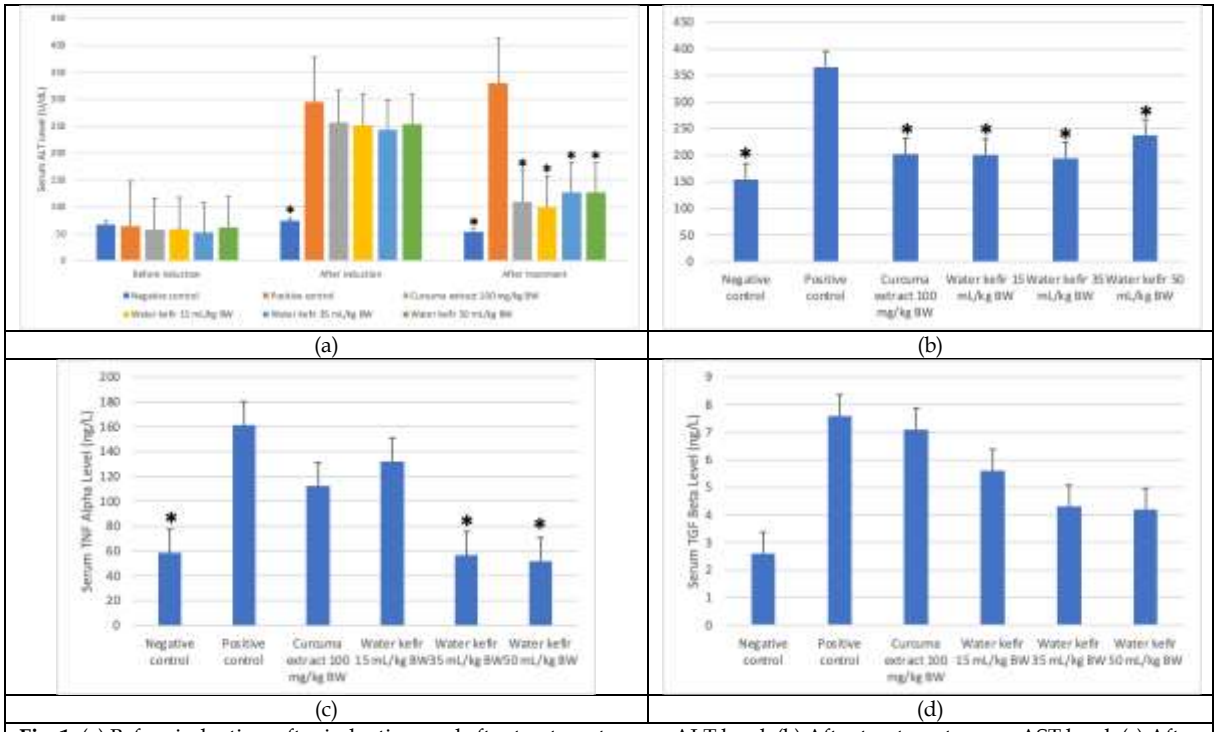


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF-α level; (d) After treatment serum TGF-β level. * indicates a significantly different result when compared to the positive control group; # indicates a significantly different result when compared to the curcuma extract group; $p < 0.05$; n = 4 mice in each group.

A histological examination of a normal liver group revealed a typical central vein bordered with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct cell borders and sinusoidal gaps (Fig 2A & 2B). The CCl4-induced group developed centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The

group that received either curcuma extract or water kefir treatment improved in varied necrotic conditions ranging from level 1 (water kefir 50 mL/ kg BW) (Fig 2K & 2L) to level 2 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Fig 2G & 2H), water kefir 30 mL/kg BW (Fig 2I & 2J)).

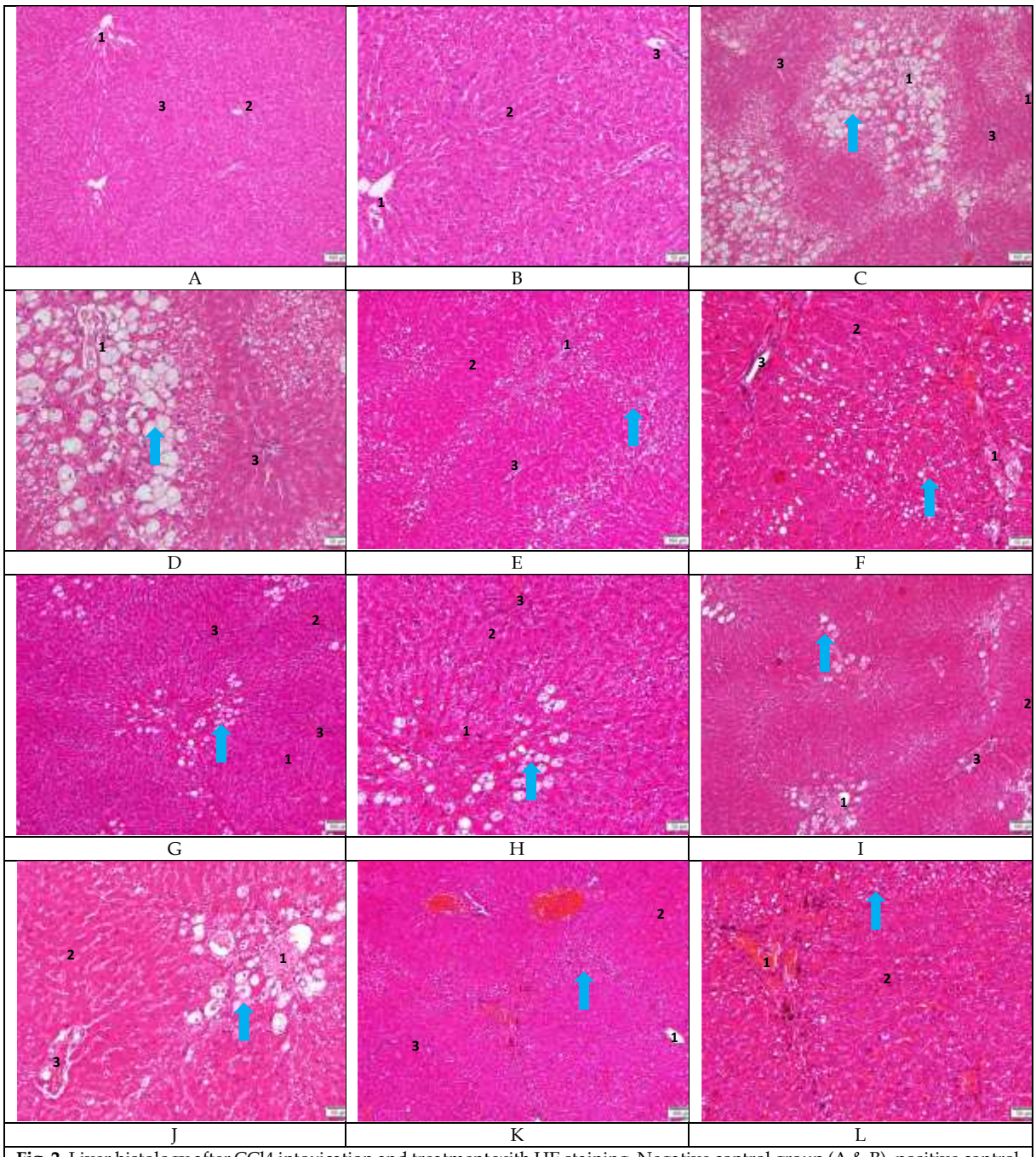
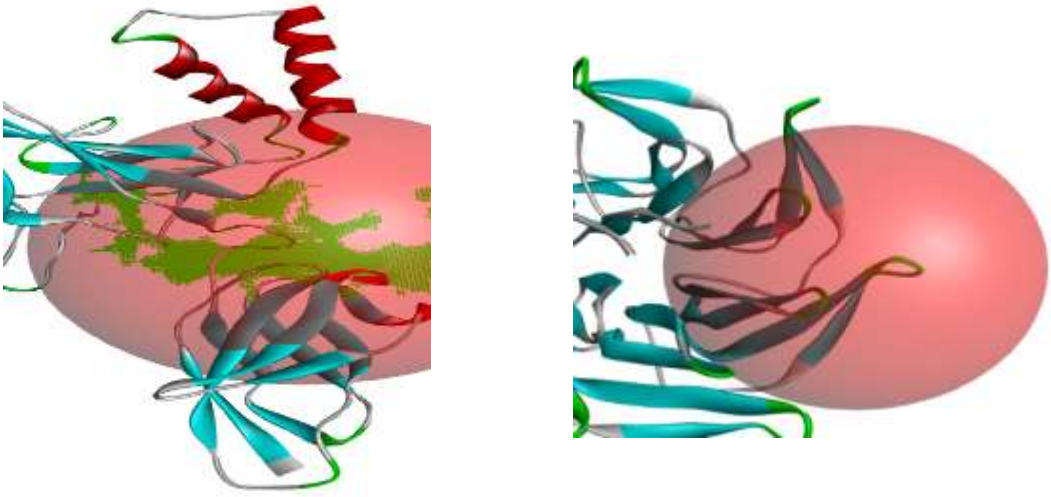


Fig. 2. Liver histology after CCl4 intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal liver epithelial cell; 3: portal track; blue arrow: necrosis and liver epithelial cell vacuolization at centrolobular region.

Molecular docking

Molecular docking studies are considered a powerful tool for predicting the potential targets of bioactive molecules. In order to carry out molecular docking simulations, one of the most critical steps is to identify the target active site. If the target protein is crystallized with a

native ligand, in many instances, the location of the active site can be established without any difficulty (32). However, the NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) proteins do not have a native ligand, so the active site was determined. Active site prediction in docking is a computational method for predicting the location and orientation of a receptor protein's binding site for a ligand molecule. The active site prediction is based on a protein structural analysis and the identification of amino acid residues that are likely to interact with the ligand. The projected binding site is then utilized as a starting point for molecular docking, a computer method for predicting a ligand molecule's binding affinity and orientation to a receptor protein. The active site prediction for target proteins (Keap1 and NF-κB) gives the grid box coordinates (x y z) of 17.500880, 62.323000, and 0.973748; and -1.751769, -20.743853, and -29.010438, respectively (Fig. 3).



A B

Fig. 3. Binding pocket (red color) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF-κB (PDB ID 1A3Q) B) Keap1 (PDB ID 4L7B).

The docking results of the 25 metabolites could interact with target proteins (Keap1 and NF-κB) (Table 1). In general, all metabolites were able to form interactions with both NF-κB receptors (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). For volatile compounds, 2-phenyl ethanol and benzaldehyde interact most strongly with the NF-κB receptor (PDB ID 1A3Q) with almost the same binding energy of -4.19 kcal/mol. As for organic acids, succinic acid, fumaric acid, and citric acid provide nearly the same strong interactions. Bond energy values of succinic acid, fumaric acid, and citric acid of -6.24, -6.66, and -6.25 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the NF-κB receptor (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2 Keap1 receptor (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to interact most strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46 kcal/mol. As for organic acids, succinic acid provides nearly the same strong interaction with a bond energy value of -3.02 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-κB (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B)

No.	Metabolites		PDB: 1A3Q		PDB: 4L7B	
			Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)	Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)

	Volatile compounds					
1	2-Methylbutanol		-3.31	3,770	-2.68	10,890
2	3-Methylbutanol		-3.22	4,360	-2.50	14,670
3	2-Phenylethanol		-4.19	851.52	-3.12	5,140
4	Ethyl acetate		-3.19	4,590	-2.43	16,670
5	2-Methylbutanal		-3.26	4,050	-2.35	19,050
6	1-Octanol		-2.65	11,490	-2.11	28,220
7	2-Phenylethyl acetate		-3.99	1,190	-3.33	3,590
8	Benzyl acetate		-4.16	887.66	-3.44	3,020
9	Benzaldehyde		-4.19	853.74	-3.19	4,550
10	Furfuryl acetate		-3.60	2,320	-3.32	3,660
11	Isobutyl acetate		-3.19	4,610	-2.94	7,020
12	Isopentyl acetate		-3.20	4,510	-3.05	5,820
13	2-Phenylacetaldehyde		-4.07	1,030	-3.46	2,930
14	3-Methylbutanal		-3.16	4,850	-2.73	10,020
15	Gluconic acid		-3.11	5,280	-1.63	64,290
	Organic acids					
16	Lactic acid		-4.92	249.28	-2.06	30,680
17	Succinic acid		-6.24	26.68	-3.02	6,140
18	Fumaric acid		-6.66	13.22	-2.45	16,100
19	Citric acid		-6.25	26.18	-1.87	42,440
20	Malic acid		-5.65	72.69	-2.53	13,860
21	Acetic acid		-4.99	219.33	-2.44	16,280
22	Ethanol		-2.39	17,800	-2.08	29,800
	Sugars					
23	Sucrose		-1.40	93,710	-1.33	105,320
24	Glucose		-3.71	1,900	-3.09	5,440
25	Fructose		-3.21	4,400	-2.65	11,440
	Please, it is mandatory to add a reference NF-kB inhibitor/activator.					

The theoretical binding modes of the top three metabolites with their target proteins (Keap1 and NF-κB) were shown in Fig. 3 and 4, respectively. The molecular docking results suggested that these metabolites anchor in Keap1 and NF-κB to form a complex through hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site of NF-κB was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid residues of PRO223 and LYS252, and LYS252, respectively. The interaction of benzaldehyde with the active site of NF-κB was formed by a hydrogen bond with the amino acid residue of LYS252. The interaction of fumaric acid with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction of glucose with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were able to be formed by six hydrogen bonds, different types of amino acid residues were involved in the interaction so that fumaric acid interacted more strongly with the active site of NF-κB (Fig. 4). Comment about reference compound and compare.

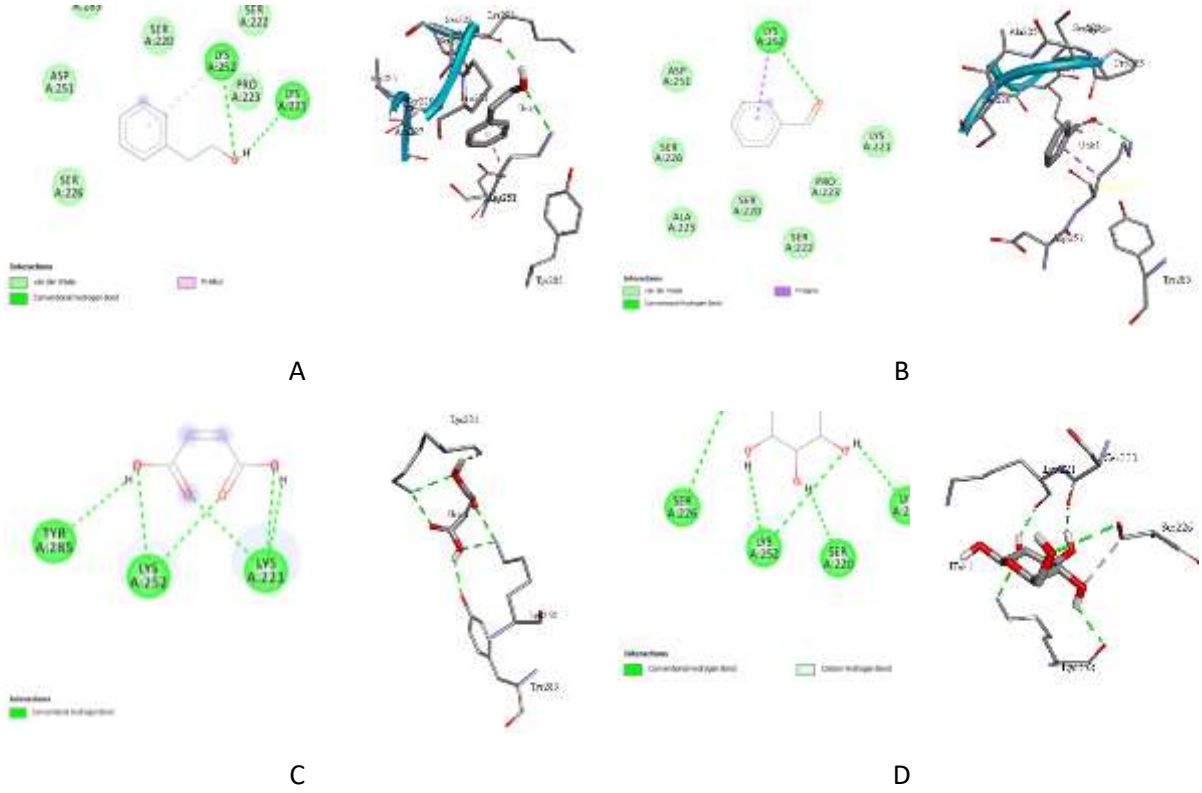


Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, and D) Glucose. Add a reference compound

248

249 The interaction of 2-phenylacetaldehyde with the active site of Keap1 was formed by one
250 hydrogen bond and one Pi-Alkyl interaction with amino acid residues of GLN530 and
251 ALA556, respectively. The interaction of succinic acid with the active site of Keap1 was formed
252 by four hydrogen bonds with the amino acid residues of ARG336, HIS575, and PHE577. The
253 interaction of glucose with the active site of Keap1 was formed by six hydrogen bonds with
254 the amino acid residues LYS551, ARG553, and ASP573. Although glucose can form more
255 hydrogen bonds than 2-phenylacetaldehyde, the different types of amino acid residues
256 involved have not been able to have a significant effect on the binding energy of its interaction
257 with the active site of Keap1 (Fig. 5).

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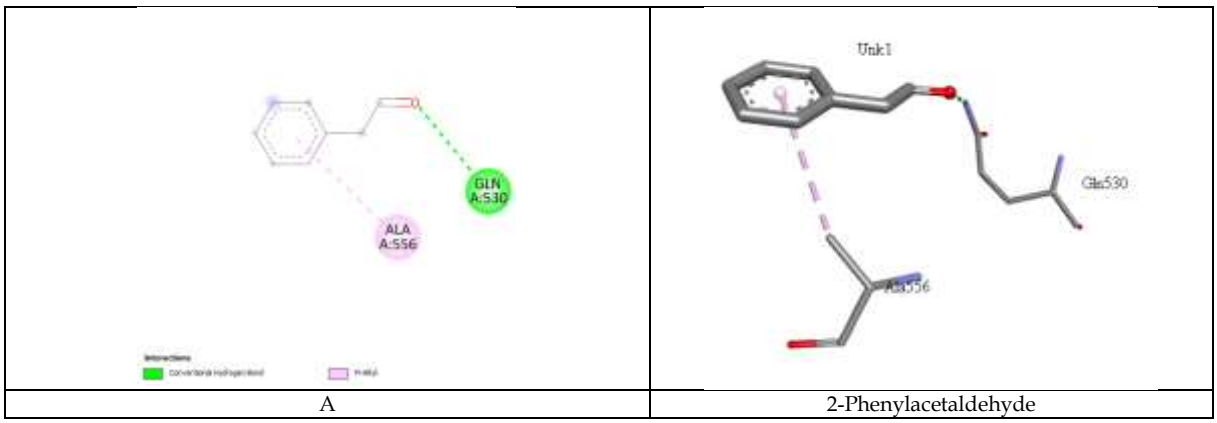
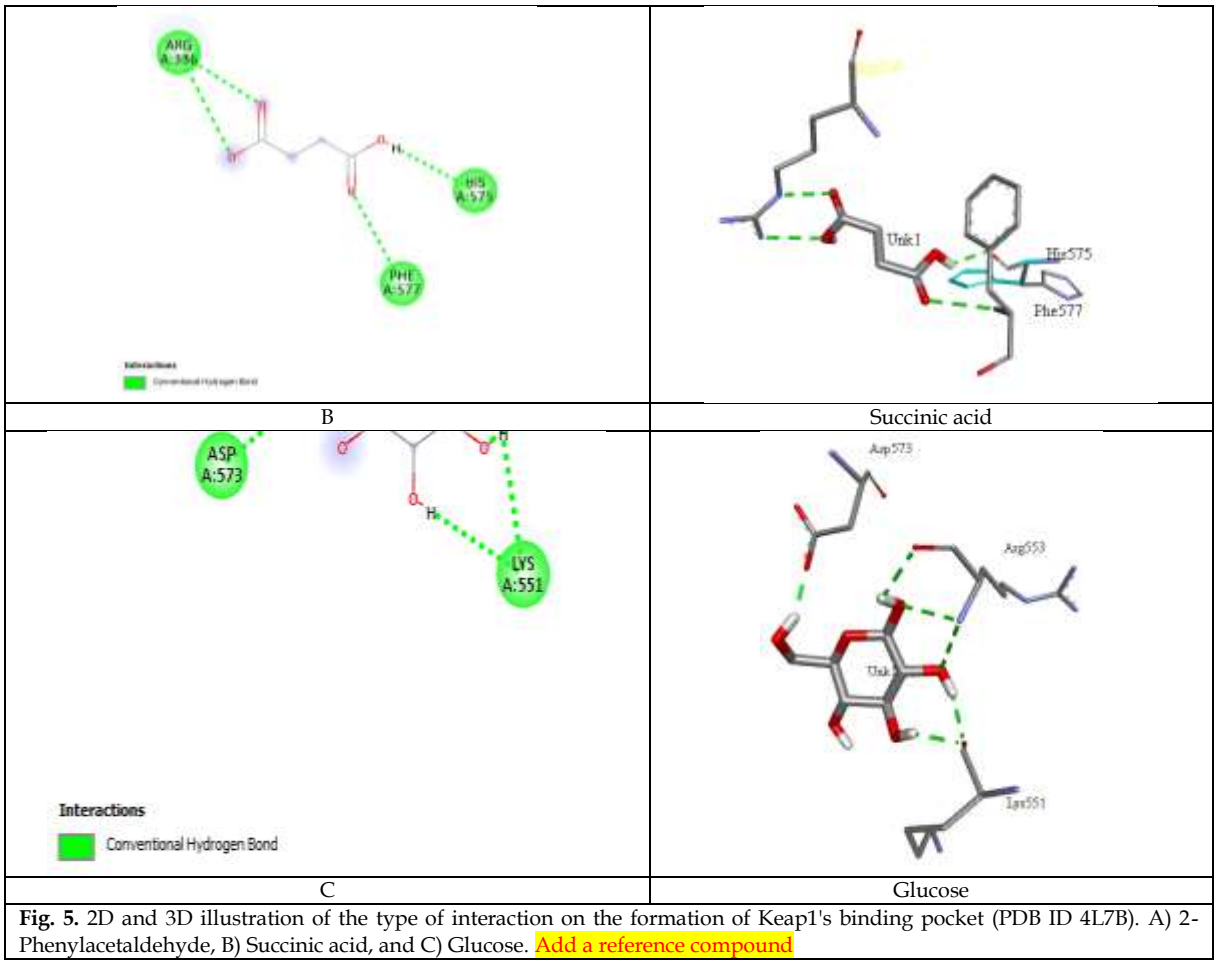


A	2-Phenylacetaldehyde



B	Succinic acid
C	Glucose

Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B). A) 2-Phenylacetaldehyde, B) Succinic acid, and C) Glucose. Add a reference compound

Overall, the metabolites of water kefir prefer to interact with NF-κB and nrf2 Keap1 receptors. Where, fumaric acid and 2-phenylacetaldehyde are metabolites that have the strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

DISCUSSION

Increased liver enzyme production is one of the abnormalities indicating liver damage. This increase in secretion increases the leakage of enzymes into the circulation. Transaminase is a frequent enzyme used to evaluate liver disorders. The transaminases AST and ALT catalyze the transfer of the α-amino group from alanine and aspartic acid to α-ketoglutaric acid. AST is found in the liver, heart, and skeletal muscle, as well as the kidney, brain, pancreas, lung, leukocytes, and erythrocytes. While ALT is dominantly found in the liver, although it is also found in low concentrations in other tissues (33). Consequently, ALT was used as the principal hepatotoxicity criterion in this study.

Carbon tetrachloride (CCl4) is a component of the hepatotoxin, which acts after metabolic activation and is extensively employed as a liver-damaging agent. And in this study, the administration of CCl4 to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administration. CCl4 is metabolized by the enzyme cytochrome p450 (CYP2E1) in the endoplasmic reticulum, to a highly reactive trichloromethyl radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl peroxyl radical, which rapidly reacts with lipids to form lipid peroxidation products. Free radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased

protein synthesis, and increased oxidase activity. CCl4 hepatotoxicity is characterized by hepatocellular necrosis with fat deposition (34). At the molecular level, administration of CCl4 can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (35).

In terms of its pathophysiological underpinnings, liver illness is linked to a condition known as dysbiosis, which refers to an imbalance in the make-up of the gut microbiota (36–38). Both qualitative and quantitative changes in the gut microbiome have the potential to affect the composition of products produced by the microbiota, such as short-chain fatty acids and bile acids (36). Qualitative changes include an imbalance between harmful and helpful microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of microbial products can all be caused by dysbiosis (39). For this reason, the condition of the gut microbiome is an important factor in the initiation and development of chronic liver disease (40). Based on the results of the study, treatment with water kefir for 2 weeks after the occurrence of liver damage was able to improve the overall condition of the liver which was marked by a significant decrease in the values of AST, ALT, TNF-α, TGF-β, and significant improvement in liver histology.

Water kefir contains a number of microorganisms that have been linked to health benefits, such as the probiotics *L. paracasei* and *B. cereus* (41). In several animal models of liver injury, *L. paracasei* was able to ameliorate liver abnormalities. Increased levels of antioxidants like glutathione and catalase are linked to this activity, as is the downregulation of pro-inflammatory transcription factors such as nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4. Improvements in intestinal barrier function and histological integrity were also observed. Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs simultaneously with the restoration of the p38 MAPK pathway (41–44). Bacillus is a kind of endospore-forming bacterium that can endure extremely cold temperatures and lengthy periods of storage without dying; its spores can even tolerate the acidic environment of the stomach and make it all the way to the small intestine (45). *B. cereus* has been shown to reduce ALT levels, an indicator of liver healing, in various animal models of liver injury. It protects the liver by reducing inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines (46–48). In addition, hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC were significantly reduced after pretreatment with *Bacillus spores*. This effect is comparable to that of the popular hepatoprotective compound silymarin (49).

Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria apart from others is their alkaline-stable lipid membrane (50). Their "oxidative" fermentation metabolism is responsible for the principal metabolic process in these bacteria, the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond, fermentation helps mediate the transition of diverse substrates into products. Although lactic acid bacteria have been studied more extensively than acetic acid bacteria (51,52), various studies have shown promising results concerning the pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the severity of liver injury in rats with septic shock by increasing the expression of TRIM40. TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of cytokines such as IL-6 and TNF-α, raise the expression of IL-10, improve survival in septic mice, and block the activity of the TLR4 signaling pathway.

Acetic acid injection decreased inflammation as well as the production of inflammatory cytokines (53). Acetic acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation and reduced hepatic lipid and body fat deposition (54,55).

Apart from microorganisms that directly provide hepatoprotective effects, the metabolites produced from these microorganisms also have the potential as hepatoprotective. Molecular docking is a technique that is utilized in the context of NF-κB and Nrf2 to make predictions regarding the binding affinity and orientation of small-molecule inhibitors to their active site. The transcription factor known as NF-κB is an essential component in the management of both the immune system and the inflammatory response (56). The expression of important inflammatory genes can be inhibited by small-molecule inhibitors that impair the interaction between NF-κB and DNA. These inhibitors have the potential to be used in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (57,58). Small-molecule inhibitors that disrupt the link between Keap1 and Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be disrupted by small-molecule inhibitors (59).

Binding energy and Ki are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor protein. Binding energy is the energy released when a ligand binds to a receptor protein, and it is calculated as the difference between the energy of the bound complex and the energy of the unbound ligand and protein (bibliography). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex, which represents the concentration of the ligand required to occupy 50% of the receptor binding sites. Both binding energy and Ki are used to predict the binding affinity and selectivity of a ligand to a receptor protein (bibliography). Molecular docking studies often report the binding energy and Ki values of the docked ligand-receptor complex to evaluate the strength of the interaction and to compare the binding affinity of different ligands (bibliography).

Based on the results of an in silico study of water kefir metabolite compounds, it was known that fumaric acid and 2-phenylacetaldehyde have strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively (bibliography). Fumaric acid has been studied for its potential as a hepatoprotection. Fumaric acid protected rat livers against cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic injury against cadmium in rats (60). Fumaric acid esters were found to ameliorate inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic fat accumulation (61).

NF-κB and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF-κB is involved in the inflammatory response, Nrf2 is involved in the antioxidant response (62,63). Both transcription factors have been investigated as potential targets for the development of hepatoprotective agents (62,64–66). There is evidence of crosstalk between the Nrf2 and NF-kB pathways (63,66). The Nrf2 pathway inhibits the activation of the NF-κB pathway by increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and detoxifies (63). The crosstalk between Nrf2 and NF-κB could be a new therapeutic target against hepatotoxicity (66). Researchers have tried to identify molecule activators of Nrf2 as chemoprevention ROS-dependent carcinogenesis, while others have focused on identifying Nrf2 inhibitors to increase sensitivity of cancer cells to chemotherapy (67). While NF-κB and Nrf2 are involved in different cellular

processes, they have both been investigated as potential targets for the development of hepatoprotective agents. Molecular docking studies have been used to investigate the interaction of potential hepatoprotective agents with these transcription factors. There is also evidence of crosstalk between the Nrf2 and NF-κB pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, the NF-κB plays a crucial role in regulating inflammation and cell death (bibliography). In response to many stimuli that may pose a threat to the host, NF-κB is activated, setting in motion processes such as inflammation, immunity, wound healing, and pathogen clearance (bibliography). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs) include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory cytokines including tumor necrosis factor (TNF) and interleukins (IL)-1 (bibliography). Numerous genes containing κB-binding sites are transcribed in response to NF-κB activation; these genes play important roles in regulating inflammation, the immunological response, and cell survival. In an NF-κB-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by reducing BAMBI expression (an inhibitory TGF-β pseudoreceptor) and increasing Kupffer cell chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF-β, which stimulates HSCs unrestrictively. When HSCs have been activated, NF-κB serves a second crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine way, all play roles in activating NF-κB in activated hepatic stellate cells. More activated HSCs and extracellular matrix are deposited in the liver as a result of greater HSC activation and survival (68).

TNF-α and TGF-β can stimulate one another's production, and TNF-α has been shown to influence TGF-β expression in a variety of cells and tissues (69). TNF-α is an inflammatory cytokine that contributes to liver inflammation, and chronic liver inflammation results in liver fibrosis. TNF-α exerts its effects on liver fibrosis via multiple mechanisms, including TGF-β signaling activation (70). Targeting TNF-α and TGF-β signaling pathways may, therefore, have therapeutic potential for treating liver diseases. In regards to hepatoprotective effects, the relationship between TGF-β and TNF-α is complex and not completely understood.

CONCLUSION

We evaluated the hepatoprotective effect of Indonesian water kefir on CCl4-induced liver-damaged rats. Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF, TGF, and an improvement of liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Hepatoprotective Study of Indonesian Water Kefir Against CCl_4-Induced Liver Injury in Rats

Running title: Hepatoprotective study of water kefir

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Concepts or Ideas	X	X	X	X
Design	X	X	X	X
Definition of intellectual content	X	X	X	X
Literature search	X			X
Clinical trial				
Experimental studies	X			X
Data acquisition	X			X
Data analysis	X			X
Statistical analysis	X			X
Manuscript preparation	X	X	X	X
Manuscript editing	X	X	X	X
Manuscript review	X	X	X	X

ABSTRACT

Context: Water kefir is a fermented beverage that is typically made in the home by inoculating a sugar-rich solution with a microbial community (water kefir grains). Several studies on the metabolite content and hepatoprotective effects of water kefir have been published, but CCl_4-induced acute liver injury has not been studied.

Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl_4-induced acute liver injury and to in silico investigate metabolites that play an important role in hepatoprotective mechanisms.

Methods: The present study aimed to investigate the hepatoprotective activity of water kefir in an animal model caused by carbon tetrachloride (CCl4). Furthermore, using molecular docking, the metabolites found in water kefir were evaluated for their role in the NF-κB and Nrf2 signaling pathways.

Results: Water kefir significantly and dose-dependently alleviated acute liver injury caused by carbon tetrachloride (CCl4). Water kefir administration at all doses produced results comparable to the positive control (Curcuma extract). Molecular docking simulations showed that, compared to Nrf2, the 25 metabolites were more likely to interact with the NF-B receptor. Fumaric acid is the strong metabolite that interacts with the NF-κB receptor with a free energy of binding and an inhibition constant of -6.66 kcal/mol and 13.22 μM, respectively.

Conclusions: Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF-α, TGF-β, and an improvement in the liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

Keywords: Free radicals; Liver diseases; Kefir; Molecular docking simulation; Probiotics

INTRODUCTION

In most cases, making water kefir involves combining dried fruit, sugar, and water kefir grains in a container. Water kefir's exact origins are unknown; however, two hypotheses have been proposed regarding its history: the first suggests that water kefir grains were brought to Europe from the Caucasus by soldiers returning from the Crimean War in the late nineteenth century (Ward 1892); the second theory proposes that water kefir grains originated in Mexico from the *Opuntia cactus* through natural processes (Moinas et al. 1980). Sugary kefir grains, Balm of Gilead, African bees, Japanese beer seeds, Ale nuts, and California bees are some other names for water kefir. Tibi grains and ginger beer plants are other names for water kefir (Kebler 1921; Moinas et al. 1980; Pidoux 1989). Water kefir is appealing to both consumers and researchers due to the variety of microbiota it contains, the fact that it is an alternative to dairy products, the versatility with which it can be flavored, the fact that it is low in calories and sugar, the ease with which it can be produced, and the health benefits it offers.

Water kefir has been used medicinally for a very long time, and recent research has indicated that it may have a variety of positive effects on people's health. It has been demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*, *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus ochraceus, A. niger, A. flavus, Penicillium sp.*, and *Rhizopus sp.* (Al-Mohammadi et al. 2021). In addition to its antibacterial properties, water kefir possesses a broad spectrum of pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al. 2003; Aligita et al. 2020), antioxidant (Alsayadi et al. 2013; Aligita et al. 2020; Darvishzadeh et al. 2021), hepatoprotective (Aspiras et al. 2015; Aligita et al. 2021), antihyperglycemic and antihyperlipidemic (Alsayadi et al. 2014; Rocha-Gomes et al. 2018), anti-edematous (Moreira et al. 2008), antitumor (Zamberi et al. 2016), antihypertensive (Gamba et al. 2019), immunomodulant (Calatayud et al. 2021), and anti-ulcerogenic (Rodrigues et al. 2016). However, no studies have been reported on the hepatoprotective effects of water kefir against carbon tetrachloride (CCl_4)-induced liver injury.

Studies have shown that acute liver injury is frequently accompanied by high levels of oxidative stress and inflammatory responses (Dai et al. 2021a). These findings have been found in several studies. The most important signaling pathways that are involved in the regulation of inflammation and antioxidation are the nuclear factor (NF-κB) and nuclear factor erythroid

2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and inhibiting NF-κB can reduce the amount of damage done to the liver. For instance, curcumin protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1) (Wang et al. 2022b). Also, it has been shown that the molecule methoxy eugenol, which comes from nutmeg and Brazilian red propolis, protects the liver both *in vitro* and *in vivo*. This may be attributed to the fact that it targets the NF-κB signaling pathway, which has been shown to have anti-inflammatory effects (De Souza Basso et al. 2021).

Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage. Additionally, a variety of aromatic and volatile compounds are produced, including ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others (compared to their threshold levels) (Laureys and De Vuyst 2014). The chemical constituents of both phytochemicals and secondary metabolites in natural products, including water kefir, are certainly capable of providing various pharmacological effects for the body (A. Asnawi et al. 2022; Nursamsiar et al. 2022). However, an *in silico* study to evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity to speed up the process of identifying and optimizing lead compounds, the *in silico* method has become the front-runner in the race to improve the speed and accuracy of the process of discovering new drugs. This is because the *in silico* method can identify and optimize lead compounds more quickly. Techniques such as molecular docking and molecular dynamics (MD) were able to directly indicate a small number of compounds that have high affinity and selectivity by analyzing how the ligand and target interact with one another (Febrina et al. 2021).

Water kefir has been used for an extensive period of time and has been recognized for its widespread benefits, especially in Indonesia. However, its level of popularity falls short in comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl_4-induced rats while also investigating the stability interactions of its metabolites within the NF-κB and Nrf2 receptors using molecular docking studies.

MATERIAL AND METHODS

Materials and Reagents

Water kefir grains, sugar, dried fruit, carbon tetrachloride, rats, diagnostic kits for alanine aminotransferase (ALT) (Proline, IFCC mod.), aspartate aminotransferase (AST) (Proline, IFCC mod.), Elisa Kit TNF-α (Bioassay Technology Laboratory), Elisa Kit TGF-β (Bioassay Technology Laboratory). Other chemicals used in this study were of analytical reagent grade.

Experimental sample and reference extract

The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution was produced using a fermentation procedure. The initial stage involved the preparation of 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The sugar and warm distilled water were mixed in a beaker, followed by the addition of water kefir grains and raisins to the resulting sugar solution. The fermentation procedure was conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

to cover the beaker glass. The kefir grain was utilized in future production, while the filtrate was employed for the purpose of evaluation and analysis. (Aligita et al. 2020, 2021)

The rhizoma extract of Curcumae (Curcuma Xanthorrhiza Roxb) is employed as a reference drug. The utilized product is a standardized herbal medicine with the brand name Tulak, manufactured by the Borobudur Company Herbal Medicine Industry. The primary purpose of Tulak capsules is to support and preserve optimal liver functionality.

Animals and Experimental Design

Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water *ad libitum*. Four mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) with the ethical approval number 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats received CCl_4 (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al. 2011). The rats were randomized into five groups after the development of animals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with the addition (6) negative control group. Each group received group-specific treatment for two weeks, along with the administration of 1.25 mL/kg BW of CCl_4 (20% in olive oil) every three days.

The rats, which had undergone a fasting period of 8–10 hours while being provided with water, had their blood samples collected via the retro-orbital sinus using a hematocrit capillary tube. The blood sample was taken into a microtube and afterwards then to centrifugation. The serum was separated in order to facilitate further measurements (Parasuraman et al. 2010). Serum ALT level, as the main parameter, was measured prior to induction, following induction, and following treatment. Meanwhile, following therapy, serum AST, TNF-α, TGF-β levels, and liver histopathological were evaluated. The ALT, AST, TNF-α, TGF-β levels measurements are conducted in accordance with the protocols outlined in the reagent kit.

After the euthanasia procedure, the liver specimen was promptly immersed in a 10% formalin solution at room temperature for a duration of 24 hours. Subsequently, the tissues were immersed in paraffin, subjected to sectioning, and affixed onto glass microscope slides. The slices underwent staining with hematoxylin and eosin and were afterwards analyzed using light microscopy (Konstantopoulos et al. 2017).

Molecular docking simulation

Molecular docking experiments were done with the PyRx software (Dallakyan and Olson 2015) to predict how metabolites, which are small-molecule ligands, bind to biological macromolecules. The NCBI PubChem database (https://pubchem.nlm.nih.gov/, accessed on 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites (Patel et al. 2022). Target proteins like NF-κB (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/, accessed on 03 May 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et al. 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study (Febrina et al. 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the protein and ligand complex and distance (Ischak et al. 2023). The BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

Statistical Analysis

All of the information is displayed in the form of individual data points as well as the mean along with the standard error of the mean. The statistical analysis was carried out with the help of Minitab software (version 19.0), and to make comparisons between several different groups, a one-way analysis of variance with Dunnett's post hoc test was utilized. All statistical graphs were created with Microsoft Excel 2019 in their respective versions. The levels of significance that were considered to have been reached were *$p < 0.05$.

RESULTS

In vivo evaluation of hepatoprotective activity

The serum ALT levels, as the main parameter for the liver damage, were measured prior to and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment AST, TNF-α, and TGF-β levels were also evaluated. These findings were analyzed using a one-way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant rise in blood ALT levels demonstrated the establishment of an animal model with liver injury, according to statistical analysis, following the administration of CCl_4. When compared to the positive control group, ALT serum levels decreased significantly after two weeks of therapy with curcuma extract or water kefir. The three doses of water kefir groups demonstrated equivalent activity when curcuma extract was used as the standard treatment, and there was no significant difference between the three doses of water kefir. When compared to the positive control group, AST levels were also reduced dramatically following treatment with curcuma extract or water kefir. TNF-α levels in the water kefir group were significantly lower than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though there was no statistically significant difference in TGF-β levels, the group that received the treatment demonstrated a decrease in TGF-β levels.

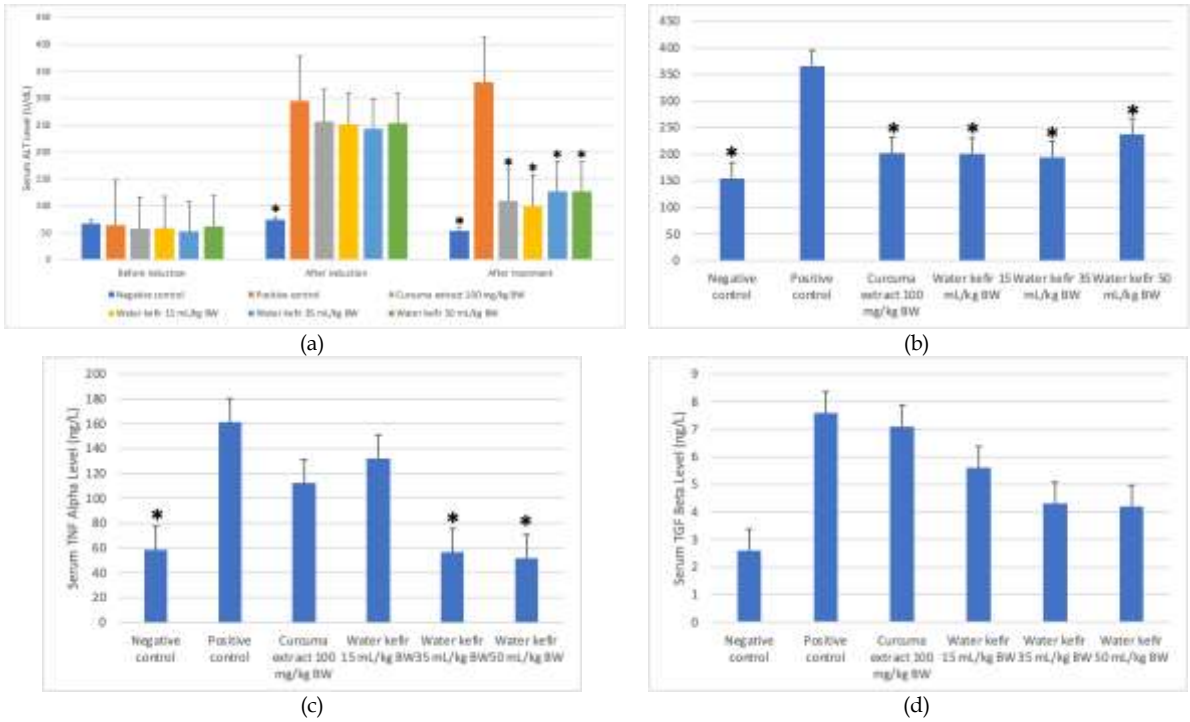


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF-α level; (d) After treatment serum TGF-β level. * indicates a significantly different result when compared to the positive control group; # indicates a significantly different result when compared to the curcuma extract group; $p < 0.05$; n = 4 mice in each group.

A histological examination of a normal liver group revealed a typical central vein bordered with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl4-induced group developed centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The group that received either curcuma extract or water kefir treatment improved in varied necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir 30 mL/kg BW (Fig 2I & 2J)).

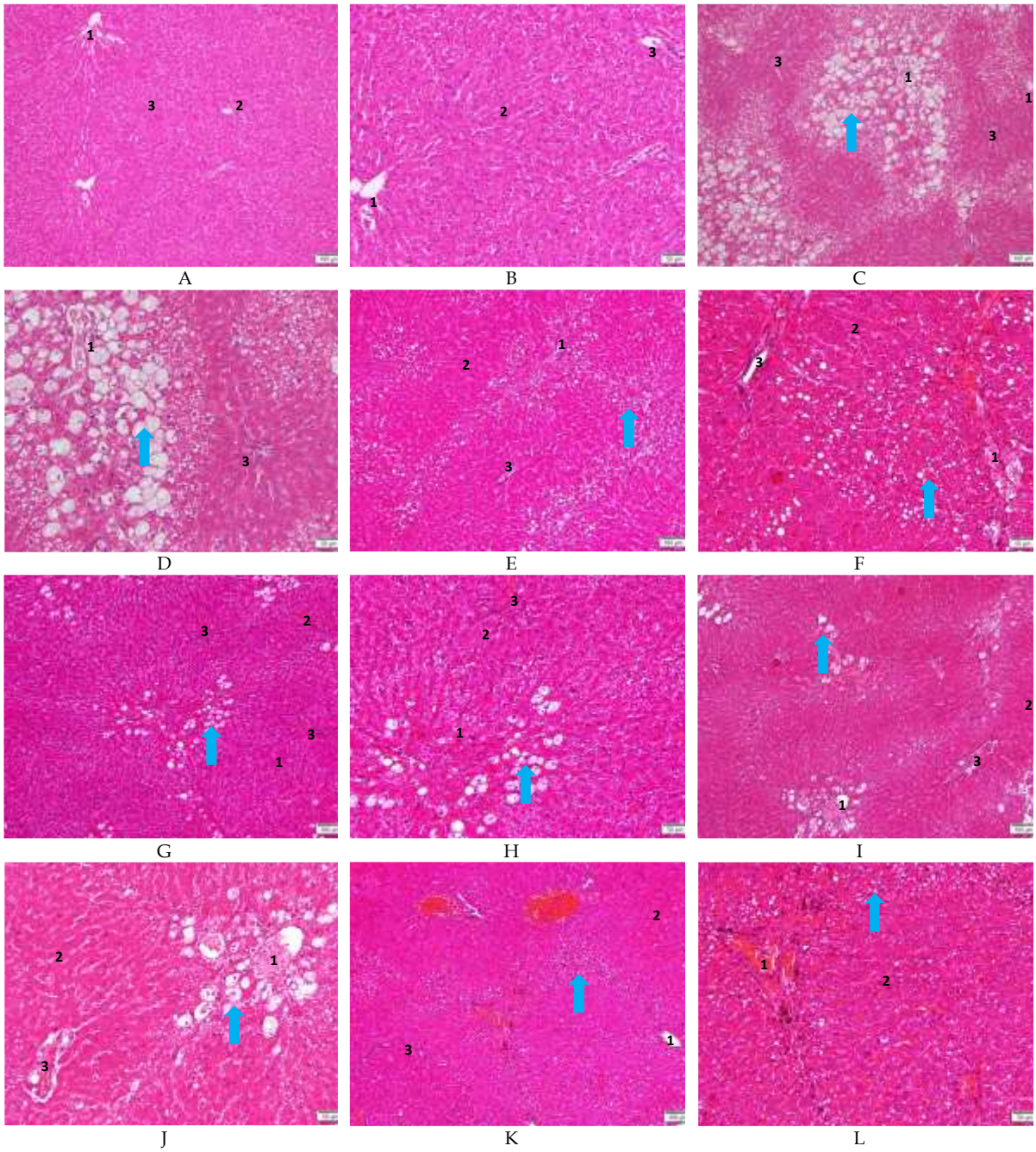
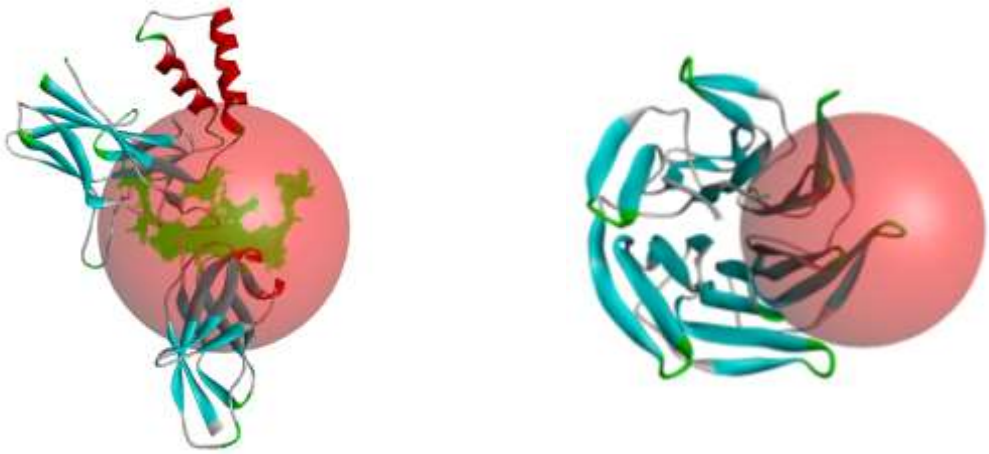


Fig. 2. Liver histology after CCl4 intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal liver epithelial cell; 3: portal track; blue arrow: necrosis and liver epithelial cell vacuolization at centrolobular region.

Molecular docking

Molecular docking studies are considered a powerful tool for predicting the potential targets of bioactive molecules. In order to carry out molecular docking simulations, one of the most critical steps is to identify the target active site. If the target protein is crystallized with a native ligand, in many instances, the location of the active site can be established without any difficulty (Li et al. 2019b). However, the NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) proteins do not have a native ligand, so the active site was determined. Active site prediction in docking is a computational method for predicting the location and orientation of a receptor protein's binding site for a ligand molecule. The active site prediction was based on a protein structural analysis and the identification of amino acid residues that are likely to interact with the ligand. The projected binding site is then utilized as a starting point for molecular docking, a computer method for predicting a ligand molecule's binding affinity and orientation to a receptor protein. The active site prediction for target proteins (Keap1 and NF-κB) gives the grid box coordinates (x y z) of 17.500880 Å, 62.323000 Å, and 0.973748 Å; and -1.751769 Å, -20.743853 Å, and -29.010438 Å, respectively (Fig. 3).



A B

Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF-κB (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

The docking results of the 25 metabolites could interact with target proteins (Keap1 and NF-κB) (Table 1). In general, all metabolites could interact with both NF-κB receptors (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). The interaction of metabolites with nuclear factor-kappa B receptors (PDB ID 1A3Q) revealed that 13 metabolites possessed free energy for binding that was greater than that of curcumin. On the other hand, there was not a single metabolite that had a free energy binding that was more powerful than that of nrf2 Keap1 (PDB ID 4L7B) (Table 1).

For volatile compounds, 2-phenyl ethanol and benzaldehyde interact most strongly with the NF-κB receptor (PDB ID 1A3Q) with almost the same binding energy of -4.19 kcal/mol. As for organic acids, succinic acid, fumaric acid, and citric acid provide nearly the same strong interactions. Bond energy values of fumaric acid, succinic acid, and citric acid of -6.66, -6.24, and -6.25 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the NF-κB receptor (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2 Keap1 receptor (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to interact most strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46 kcal/mol. As for organic acids, succinic acid provides nearly the same strong interaction with

a bond energy value of -3.02 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-κB (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B)

No.	Metabolites	PDB: 1A3Q Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)	PDB: 4L7B Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)
	Volatile compounds				
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-4.19	851.52	-3.12	5,140
4	Ethyl acetate	-3.19	4,590	-2.43	16,670
5	2-Methylbutanal	-3.26	4,050	-2.35	19,050
6	1-Octanol	-2.65	11,490	-2.11	28,220
7	2-Phenylethyl acetate	-3.99	1,190	-3.33	3,590
8	Benzyl acetate	-4.16	887.66	-3.44	3,020
9	Benzaldehyde	-4.19	853.74	-3.19	4,550
10	Furfuryl acetate	-3.60	2,320	-3.32	3,660
11	Isobutyl acetate	-3.19	4,610	-2.94	7,020
12	Isopentyl acetate	-3.20	4,510	-3.05	5,820
13	2-Phenylacetaldehyde	-4.07	1,030	-3.46	2,930
14	3-Methylbutanal	-3.16	4,850	-2.73	10,020
15	Gluconic acid	-3.11	5,280	-1.63	64,290
	Organic acids				
16	Lactic acid	-4.92	249.28	-2.06	30,680
17	Succinic acid	-6.24	26.68	-3.02	6,140
18	Fumaric acid	-6.66	13.22	-2.45	16,100
19	Citric acid	-6.25	26.18	-1.87	42,440
20	Malic acid	-5.65	72.69	-2.53	13,860
21	Acetic acid	-4.99	219.33	-2.44	16,280
22	Ethanol	-2.39	17,800	-2.08	29,800
	Sugars				
23	Sucrose	-1.40	93,710	-1.33	105,320
24	Glucose	-3.71	1,900	-3.09	5,440
25	Fructose	-3.21	4,400	-2.65	11,440
	Curcumin	-3.44	2,990	-4.22	811.36

The theoretical binding modes of the top three metabolites with their target proteins (Keap1 and NF-κB) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested that these metabolites interacted with the Keap1 and NF-κB to form a complex through hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site of NF-κB was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid residues of PRO223, LYS252, and LYS252, respectively. The interaction of benzaldehyde with the active site of NF-κB was formed by a hydrogen bond with the amino acid residue of LYS252. The interaction of fumaric acid with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction of glucose with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were able to be formed by six hydrogen bonds, different types of amino acid residues were involved

254 in the interaction, so fumaric acid interacted more strongly with the active site of NF-κB (Fig.
255 4).
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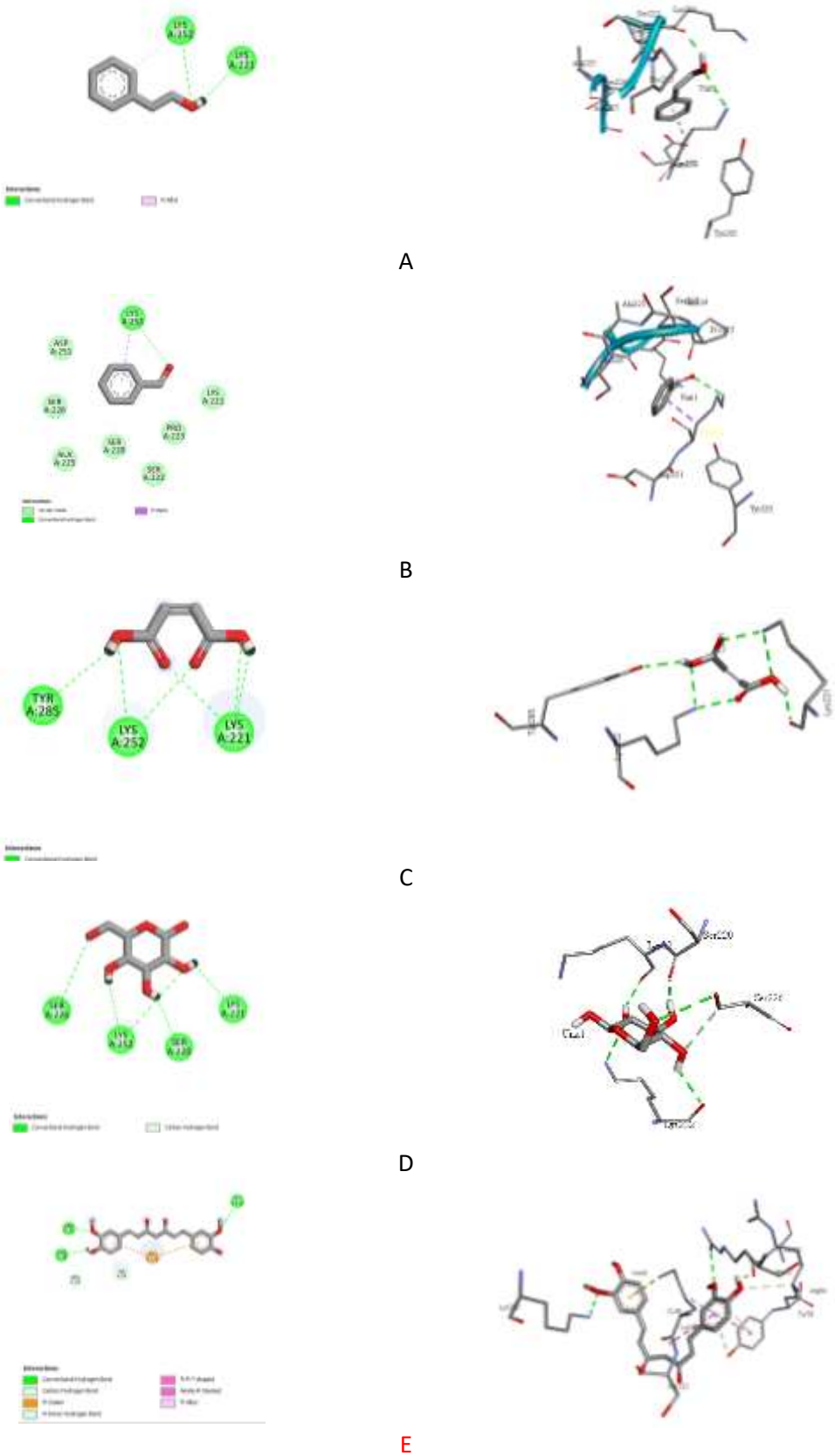

Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, D) Glucose, and E) Curcumin.

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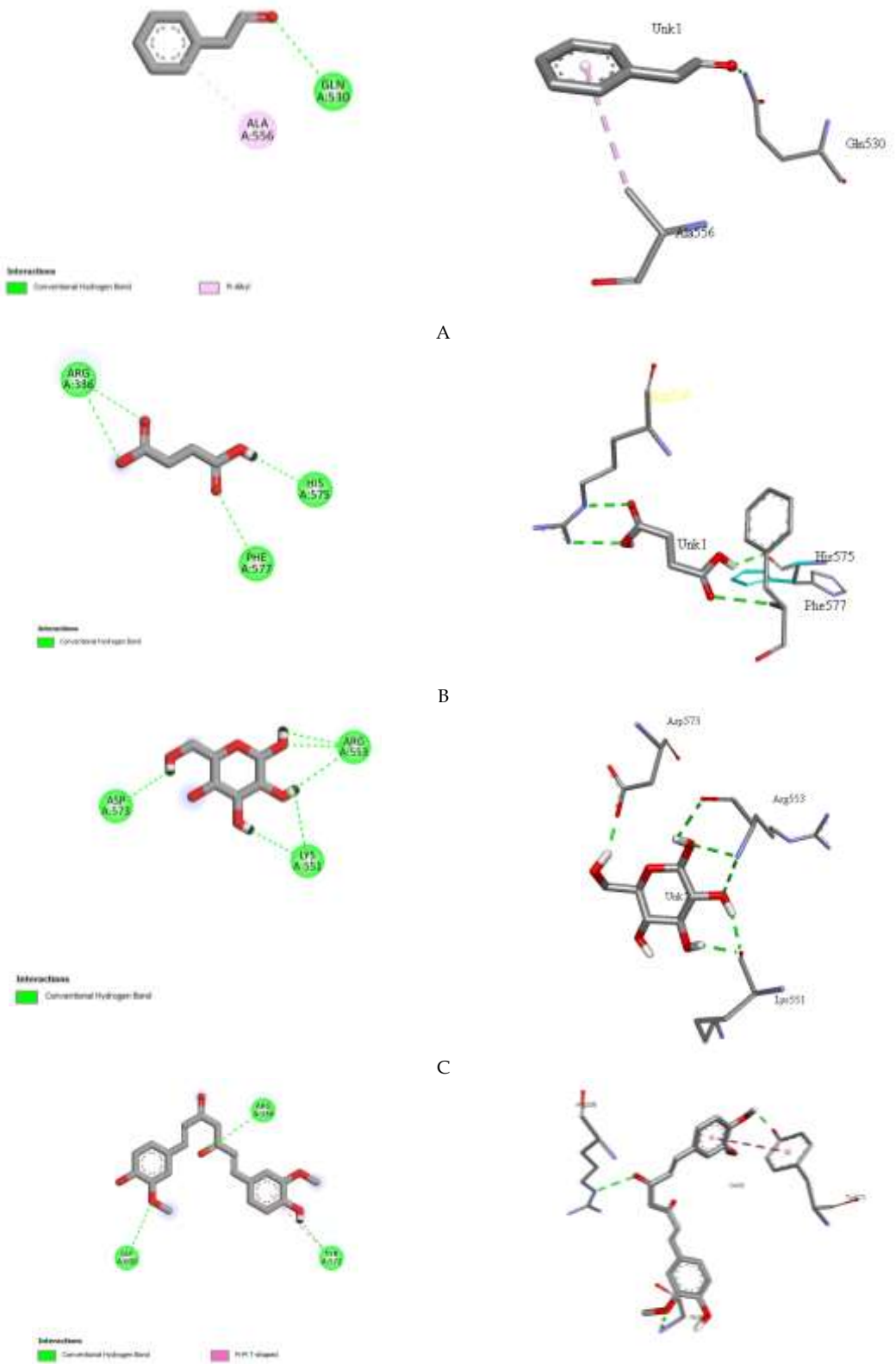

Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B). A) 2-Phenylacetaldehyde, B) Succinic acid, C) Glucose, and D) Curcumin.

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The interaction of 2-phenylacetaldehyde with the active site of Keap1 was formed by one hydrogen bond and one Pi-Alkyl interaction with amino acid residues of GLN530 and ALA556, respectively. The interaction of succinic acid with the active site of Keap1 was formed by four hydrogen bonds with the amino acid residues of ARG336, HIS575, and PHE577. The interaction of glucose with the active site of Keap1 was formed by six hydrogen bonds with the amino acid residues LYS551, ARG553, and ASP573. Although glucose can form more hydrogen bonds than 2-phenylacetaldehyde, the different types of amino acid residues involved have not been able to have a significant effect on the binding energy of its interaction with the active site of Keap1 (Fig. 5).

Curcumin (the reference compound) created three hydrogen bonds with the amino acid residues ARG52, GLU58, and LYS252 to interact with the active site of NF-κB. Meanwhile, curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572, and GLY600 to interact with the active site of nrf2 Keap1 (Figs 4 and 5). Despite the fact that curcumin could create three hydrogen bonds at both the active sites of NF-κB and nrf2 Keap1, its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol, benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to interact with NF-κB and nrf2 Keap1 receptors. Whereas, fumaric acid and 2-phenylacetaldehyde were metabolites that had the strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

DISCUSSION

Increased liver enzyme production is one of the abnormalities indicating liver damage. This increase in secretion increases the leakage of enzymes into the circulation. Transaminase is a frequent enzyme used to evaluate liver disorders. The transaminases AST and ALT catalyze the transfer of the α-amino group from alanine and aspartic acid to α-ketoglutaric acid. AST is found in the liver, heart, and skeletal muscle, as well as the kidney, brain, pancreas, lung, leukocytes, and erythrocytes. While ALT is predominantly found in the liver, it is also found in low concentrations in other tissues (Lee et al. 2012). Consequently, ALT was used as the principal hepatotoxicity criterion in this study.

Carbon tetrachloride (CCl_4) is a component of the hepatotoxin, which acts after metabolic activation and is extensively employed as a liver-damaging agent. In this study, the administration of CCl_4 to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administrations. CCl_4 is metabolized by the enzyme cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl peroxyl radical, which rapidly reacts with lipids to form lipid peroxidation products. Free radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased protein synthesis, and increased oxidase activity. CCl_4 hepatotoxicity is characterized by hepatocellular necrosis with fat deposition (Ritesh et al. 2015). At the molecular level, administration of CCl_4 can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (Weber et al. 2003).

In terms of its pathophysiological underpinnings, liver illness is linked to a condition known as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Zavala et al. 2016; Laureys and De Vuyst 2017; Romero-Luna et al. 2020). Both qualitative and quantitative changes in the gut microbiome have the potential to affect the composition of products produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et al.

2020). Qualitative changes include an imbalance between harmful and helpful microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of microbial products can all be caused by dysbiosis (Laureys et al. 2018). For this reason, the condition of the gut microbiome is an important factor in the initiation and development of chronic liver disease (Lee et al. 2021). Based on the results of the study, treatment with water kefir for 2 weeks after the occurrence of liver damage was able to improve the overall condition of the liver which was marked by a significant decrease in the values of AST, ALT, TNF-α, TGF-β, and significant improvement in liver histology.

Water kefir contains a number of microorganisms that have been linked to health benefits, such as the probiotics *L. paracasei* and *B. cereus* (Fijan 2014). This activity is linked to an increase in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4). Improvements in intestinal barrier function and histological integrity were also observed. Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs simultaneously with the restoration of the p38 MAPK pathway (Fijan 2014; Yao et al. 2019; Tsai et al. 2020; Ji et al. 2022). Bacillus is a kind of endospore-forming bacterium that can endure extremely cold temperatures and lengthy periods of storage without dying; its spores can even tolerate the acidic environment of the stomach and make it all the way to the small intestine (Elshaghabee et al. 2017). *B. cereus* has been shown to reduce ALT levels, an indicator of liver healing, in various animal models of liver injury. It protects the liver by reducing inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines (Kim et al. 2018; Li et al. 2019a; Xue et al. 2020). Also, when *Bacillus* spores were used first, hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al. 2020).

Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria apart from others is their alkaline-stable lipid membrane (Lynch et al. 2021). Their "oxidative" fermentation metabolism is responsible for the principal metabolic process in these bacteria, the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond, fermentation helps mediate the transition of diverse substrates into products. Although lactic acid bacteria have been studied more extensively than acetic acid bacteria (Semjonovs et al. 2014; Hong et al. 2021), various studies have shown promising results concerning the pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the severity of liver injury in rats with septic shock by increasing the expression of TRIM40. TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of cytokines such as IL-6 and TNF-α, raise the expression of IL-10, improve survival in septic mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased inflammation as well as the production of inflammatory cytokines (Yang et al. 2019). Acetic acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation and reduced hepatic lipid and body fat deposition (Kondo et al. 2009; Li et al. 2018).

Apart from microorganisms that directly provide hepatoprotective effects, the metabolites produced from these microorganisms also have the potential to be hepatoprotective. Molecular docking is a technique that is utilized in the context of NF-κB and Nrf2 to make predictions regarding the binding affinity and orientation of small-molecule inhibitors to their active sites. The transcription factor known as NF-κB is an essential component in the

management of both the immune system and the inflammatory response (Dai et al. 2021b). The expression of important inflammatory genes can be inhibited by small-molecule inhibitors that impair the interaction between NF-κB and DNA. These inhibitors have the potential to be used in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (Zhao et al. 2017a; Jiang et al. 2019). Small-molecule inhibitors that disrupt the link between Keap1 and Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be disrupted by small-molecule inhibitors (Zhao et al. 2017b).

Binding energy and Ki are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor protein. Binding energy is the energy released when a ligand binds to a receptor protein, and it is calculated as the difference between the energy of the bound complex and the energy of the unbound ligand and protein (Meng et al. 2011). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites. Both binding energy and Ki are used to predict the binding affinity and selectivity of a ligand to a receptor protein (Du et al. 2016). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites (Du et al. 2016).

Based on the results of an in silico study of water kefir metabolite compounds, it was known that fumaric acid and 2-phenylacetaldehyde have the strongest interactions with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively. Fumaric acid has been studied for its potential as a hepatoprotective agent. Fumaric acid protects rat livers against cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic injury against cadmium in rats (Kaur et al. 2020). Fumaric acid esters were found to ameliorate inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic fat accumulation (Šilhavý et al. 2014).

NF-κB and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF-κB is involved in the inflammatory response, and Nrf2 is involved in the antioxidant response (Ganesh Yerra et al. 2013; Wang et al. 2022a). Both transcription factors have been investigated as potential targets for the development of hepatoprotective agents (Rahman et al. 2021; Gao et al. 2022; Wang et al. 2022a; Li et al. 2023). There is evidence of crosstalk between the Nrf2 and NF-kB pathways (Ganesh Yerra et al. 2013; Gao et al. 2022). The Nrf2 pathway inhibits the activation of the NF-κB pathway by increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and detoxifies (Ganesh Yerra et al. 2013). The crosstalk between Nrf2 and NF-κB could be a new therapeutic target against hepatotoxicity (Gao et al. 2022). Researchers have tried to identify molecule activators of Nrf2 as chemoprevention for ROS-dependent carcinogenesis, while others have focused on identifying Nrf2 inhibitors to increase the sensitivity of cancer cells to chemotherapy (Sharifi-Rad et al. 2023). While NF-κB and Nrf2 are involved in different cellular processes, they have both been investigated as potential targets for the development of hepatoprotective agents. Molecular docking studies have been used to investigate the interaction of potential hepatoprotective agents with these transcription factors. There is also evidence of crosstalk between the Nrf2 and NF-κB pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF-κB plays a crucial role in regulating inflammation and cell death (Luedde and Schwabe 2011). In response to many stimuli that may pose a threat to the host, NF-κB is activated, setting in motion processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde and Schwabe 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs) include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory cytokines including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe 2011). When NF-B is activated, a lot of genes with B-binding sites are transcribed. These genes play important roles in controlling inflammation, the immune response, and cell survival. In an NF-κB-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by reducing BAMBI expression (an inhibitory TGF-β pseudoreceptor) and increasing Kupffer cell chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF-β, which stimulates HSCs unrestrictively. When HSCs have been activated, NF-κB serves a second crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine way, all play roles in activating NF-κB in activated hepatic stellate cells. More activated HSCs and extracellular matrix are deposited in the liver as a result of greater HSC activation and survival (Luedde and Schwabe 2011).

TNF-α and TGF-β can stimulate one another's production, and TNF-α has been shown to influence TGF-β expression in a variety of cells and tissues (Liu et al. 2022). TNF-α is an inflammatory cytokine that contributes to liver inflammation, and chronic liver inflammation results in liver fibrosis. TNF-α exerts its effects on liver fibrosis via multiple mechanisms, including TGF-β signaling activation (Yang and Seki 2015). Targeting TNF-α and TGF-β signaling pathways may, therefore, have therapeutic potential for treating liver diseases. In regards to hepatoprotective effects, the relationship between TGF-β and TNF-α is complex and not completely understood.

CONCLUSION

In this study, the hepatoprotective qualities of Indonesian water kefir in rats with CCl4-induced liver damage has evaluated. Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF, TGF, and an improvement of liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Bandung, September 19, 2023

Dear **Prof. Gabino Garrido**
Editor in Chief
Journal of Pharmacy & Pharmacognosy Research
Garval Editorial Ltda.
Antofagasta
Chile

Thank you for giving us the opportunity to submit a revised draft of our manuscript (ID: JPPRes-23-1732.R1) titled *Hepatoprotective Study of Indonesian Water Kefir Against CCl4-Induced Liver Injury* in *Rats* to *Journal of Pharmacy & Pharmacognosy Research (JPPRes)*. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable feedback on our manuscript. We are grateful to the reviewers for their insightful comments on our paper. We have been able to incorporate changes to reflect most of the suggestions provided by the reviewers. We have highlighted the changes within the manuscript.

Here is a point-by-point response to the reviewers' comments and concerns

	Comment	**Response**
Keywords	Please check if these keywords have been accepted by MeSH Browser 2022 or 2023 (https://meshb-prev.nlm.nih.gov/). If not, type keywords accepted by this MeSH Browser.	1. We have re-checked the keywords.
Introduction	1. There are numerous studies demonstrating the hepatoprotective activity of kefir. Therefore, the authors should state here the novel effect they intend to find, which has not been reported in the scientific literature. Please better state the research problem and how you intend to solve it. 2. Please move this last sentence to the Conclusions, it is not valid for an Introduction.	1. We have added the research problem 2. We have revised the last sentence
MATERIAL AND METHODS	1. Experimental sample and reference extract It is necessary to indicate here where these materials were obtained from. Indicate who identified the material. The manuscript should include references to voucher specimens of the plants (deposited in a major regional herbarium) or to the material examined, including their registration number(s). It should be mentioned which parts of the plant have been used. The GPS coordinates of the collection site of the species should also be indicated. It is mandatory that the authors indicate how both the kefir and the turmeric extract were prepared, the quality of these products, etc. 2. Please indicate how the serum was extracted in the rats? from which part of the animal and how the blood was extracted. How were AST, TNF-α, TGF-a levels measured? give details of this. How was the	We have revised the research methodology.

	liver extracted from the animals? how was the liver histopathological analysis performed.	
Results	1. Please, it is mandatory to add a reference NF-kB inhibitor/activator. 2. Comment about reference compound and compare. 3. Add a reference compound	We have revised the Results (Table 1, Figs 4 and 5)
Bibliography/References :	1. The style of the References does not meet the requirements of JPPRes. Please write the References according to the Instructions to the Author or the examples: https://jppres.com/jppres/archive/ https://jppres.com/jppres/volume-11-issue-2/ Also, all authors and DOIs must be given. Please check that all references in this section have been cited in the text and vice versa. Please check that the journals cited are prestigious in the discipline and are not predatory journals (https://www.openacessjournal.com/blog/predatory-journals-list/#I_%E2%80%93_predatory_journals). References must be write free of codes from any bibliography processor, such as Endnotes, Mendeley, Zotero, etc...2. Latest references must be used.	1. We have revised the references referring to the sample.

Thank you, and we look forward to hearing more about our manuscript.

With kind regards,

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Hepatoprotective study of Indonesian water kefir against CCl_4-induced liver injury in rats

Running title: Hepatoprotective study of water kefir

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Contribution Details (to be ticked marked (X) as applicable and add columns according to the number of authors):

Contribution	Aligita W	Singgih M	Sutrisno E	Adnyana IK
Concepts or Ideas	X	X	X	X
Design	X	X	X	X
Definition of intellectual content	X	X	X	X
Literature search	X			X
Experimental studies	X			X
Data acquisition	X			X
Data analysis	X			X
Statistical analysis	X			X
Manuscript preparation	X	X	X	X
Manuscript editing	X	X	X	X
Manuscript review	X	X	X	X

ABSTRACT

Context: Water kefir is a fermented beverage that is typically made in the home by inoculating a sugar-rich solution with a microbial community (water kefir grains). Several studies on the metabolite content and hepatoprotective effects of water kefir have been published, but CCl_4-induced acute liver injury has not been studied.

Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl_4-induced acute liver injury and to in silico investigate metabolites that play an important role in hepatoprotective mechanisms.

Methods: The present study aimed to investigate the hepatoprotective activity of water kefir in an animal model caused by carbon tetrachloride (CCl4). Furthermore, using molecular docking, the metabolites found in water kefir were evaluated for their role in the NF-κB and Nrf2 signaling pathways.

Results: Water kefir significantly and dose-dependently alleviated acute liver injury caused by carbon tetrachloride (CCl4). Water kefir administration at all doses produced results comparable to the positive control (Curcuma extract). Molecular docking simulations showed that, compared to Nrf2, the 25 metabolites were more likely to interact with the NF-B receptor. Fumaric acid is the strong metabolite that interacts with the NF-κB receptor with a free energy of binding and an inhibition constant of -6.66 kcal/mol and 13.22 μM, respectively.

Conclusions: Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF-α, TGF-β, and an improvement in the liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

Keywords: free radicals; liver diseases; kefir; molecular docking simulation; probiotics

INTRODUCTION

In most cases, making water kefir involves combining dried fruit, sugar, and water kefir grains in a container. Water kefir's exact origins are unknown; however, two hypotheses have been proposed regarding its history: the first suggests that water kefir grains were brought to Europe from the Caucasus by soldiers returning from the Crimean War in the late nineteenth century (Ward, 1892); the second theory proposes that water kefir grains originated in Mexico from the *Opuntia cactus* through natural processes (Moinas et al., 1980). Sugary kefir grains, Balm of Gilead, African bees, Japanese beer seeds, Ale nuts, and California bees are some other names for water kefir. Tibi grains and ginger beer plants are other names for water kefir (Kebler, 1921; Moinas et al., 1980; Pidoux, 1989). Water kefir is appealing to both consumers and researchers due to the variety of microbiota it contains, the fact that it is an alternative to dairy products, the versatility with which it can be flavored, the fact that it is low in calories and sugar, the ease with which it can be produced, and the health benefits it offers.

Water kefir has been used medicinally for a very long time, and recent research has indicated that it may have a variety of positive effects on people's health. It has been demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*, *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus ochraceus, A. niger, A. flavus, Penicillium sp.*, and *Rhizopus sp.* (Al-Mohammadi et al., 2021). In addition to its antibacterial properties, water kefir possesses a broad spectrum of pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al., 2003; Aligita et al., 2020), antioxidant (Alsayadi et al., 2013; Aligita et al., 2020; Darvishzadeh et al., 2021), hepatoprotective (Aspiras et al., 2015; Aligita et al., 2021), antihyperglycemic and antihyperlipidemic (Alsayadi et al., 2014; Rocha-Gomes et al., 2018), anti-edematous (Moreira et al., 2008), antitumor (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019), immunomodulant (Calatayud et al., 2021), and anti-ulcerogenic (Rodrigues et al., 2016). However, no studies have been reported on the hepatoprotective effects of water kefir against carbon tetrachloride (CCl_4)-induced liver injury.

Studies have shown that acute liver injury is frequently accompanied by high levels of oxidative stress and inflammatory responses (Dai et al., 2021a). These findings have been found in several studies. The most important signaling pathways that are involved in the regulation of inflammation and antioxidation are the nuclear factor (NF-κB) and nuclear factor

erythroid 2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and inhibiting NF-κB can reduce the amount of damage done to the liver. For instance, curcumin protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1) (Wang et al., 2022b). Also, it has been shown that the molecule methoxy eugenol, which comes from nutmeg and Brazilian red propolis, protects the liver both *in vitro* and *in vivo*. This may be attributed to the fact that it targets the NF-κB signaling pathway, which has been shown to have anti-inflammatory effects (De Souza Basso et al., 2021).

Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage. Additionally, a variety of aromatic and volatile compounds are produced, including ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others (compared to their threshold levels) (Laureys and De Vuyst 2014). The chemical constituents of both phytochemicals and secondary metabolites in natural products, including water kefir, are certainly capable of providing various pharmacological effects for the body (A. Asnawi et al., 2022; Nursamsiar et al., 2022). However, an *in silico* study to evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity to speed up the process of identifying and optimizing lead compounds, the *in silico* method has become the front-runner in the race to improve the speed and accuracy of the process of discovering new drugs. This is because the *in silico* method can identify and optimize lead compounds more quickly. Techniques such as molecular docking and molecular dynamics (MD) were able to directly indicate a small number of compounds that have high affinity and selectivity by analyzing how the ligand and target interact with one another (Febrina et al., 2021).

Water kefir has been used for an extensive period of time and has been recognized for its widespread benefits, especially in Indonesia. However, its level of popularity falls short in comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl_4-induced rats while also investigating the stability interactions of its metabolites within the NF-κB and Nrf2 receptors using molecular docking studies.

Please place a comma before the year in all citations throughout the document. See examples of already arranged citations in the Introduction.

MATERIAL AND METHODS

Materials and reagents

Water kefir grains, sugar, dried fruit, carbon tetrachloride, rats, diagnostic kits for alanine aminotransferase (ALT) (Proline, IFCC mod.), aspartate aminotransferase (AST) (Proline, IFCC mod.), Elisa Kit TNF-α (Bioassay Technology Laboratory), Elisa Kit TGF-β (Bioassay Technology Laboratory). Other chemicals used in this study were of analytical reagent grade.

Experimental sample and reference extract

The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution was produced using a fermentation procedure. The initial stage involved the preparation of 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The sugar and warm distilled water were mixed in a beaker, followed by the addition of water kefir grains and raisins to the resulting sugar solution. The fermentation procedure was conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

to cover the beaker glass. The kefir grain was utilized in future production, while the filtrate was employed for the purpose of evaluation and analysis. (Aligita et al. 2020, 2021)

The rhizoma extract of Curcumae (*Curcuma xanthorrhiza* Roxb) is employed as a reference drug. The utilized product is a standardized herbal medicine with the brand name Tulak, manufactured by the Borobudur Company Herbal Medicine Industry. The primary purpose of Tulak capsules is to support and preserve optimal liver functionality.

Animals and experimental design

Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water *ad libitum*. Four mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) with the ethical approval number 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats received CCl_4 (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al. 2011). The rats were randomized into five groups after the development of animals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with the addition (6) negative control group. Each group received group-specific treatment for two weeks, along with the administration of 1.25 mL/kg BW of CCl_4 (20% in olive oil) every three days.

The rats, which had undergone a fasting period of 8–10 hours while being provided with water, had their blood samples collected via the retro-orbital sinus using a hematocrit capillary tube. The blood sample was taken into a microtube and afterwards then to centrifugation. The serum was separated in order to facilitate further measurements (Parasuraman et al. 2010). Serum ALT level, as the main parameter, was measured prior to induction, following induction, and following treatment. Meanwhile, following therapy, serum AST, TNF-α, TGF-β levels, and liver histopathological were evaluated. The ALT, AST, TNF-α, TGF-β levels measurements are conducted in accordance with the protocols outlined in the reagent kit.

After the euthanasia procedure, the liver specimen was promptly immersed in a 10% formalin solution at room temperature for a duration of 24 hours. Subsequently, the tissues were immersed in paraffin, subjected to sectioning, and affixed onto glass microscope slides. The slices underwent staining with hematoxylin and eosin and were afterwards analyzed using light microscopy (Konstantopoulos et al. 2017).

Molecular docking simulation

Molecular docking experiments were done with the PyRx software (Dallakyan and Olson 2015) to predict how metabolites, which are small-molecule ligands, bind to biological macromolecules. The NCBI PubChem database (https://pubchem.nlm.nih.gov/, accessed on 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites (Patel et al. 2022). Target proteins like NF-κB (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/, accessed on 03 May 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et al. 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study (Febrina et al. 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the protein and ligand complex and distance (Ischak et al. 2023). The BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

Statistical analysis

All of the information is displayed in the form of individual data points as well as the mean along with the standard error of the mean. The statistical analysis was carried out with the help of Minitab software (version 19.0), and to make comparisons between several different groups, a one-way analysis of variance with Dunnett's post hoc test was utilized. All statistical graphs were created with Microsoft Excel 2019 in their respective versions. The levels of significance that were considered to have been reached were *$p < 0.05$.

RESULTS

In vivo evaluation of hepatoprotective activity

The serum ALT levels, as the main parameter for the liver damage, were measured prior to and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment AST, TNF-α, and TGF-β levels were also evaluated. These findings were analyzed using a one-way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant rise in blood ALT levels demonstrated the establishment of an animal model with liver injury, according to statistical analysis, following the administration of CCl_4. When compared to the positive control group, ALT serum levels decreased significantly after two weeks of therapy with curcuma extract or water kefir. The three doses of water kefir groups demonstrated equivalent activity when curcuma extract was used as the standard treatment, and there was no significant difference between the three doses of water kefir. When compared to the positive control group, AST levels were also reduced dramatically following treatment with curcuma extract or water kefir. TNF-α levels in the water kefir group were significantly lower than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though there was no statistically significant difference in TGF-β levels, the group that received the treatment demonstrated a decrease in TGF-β levels.

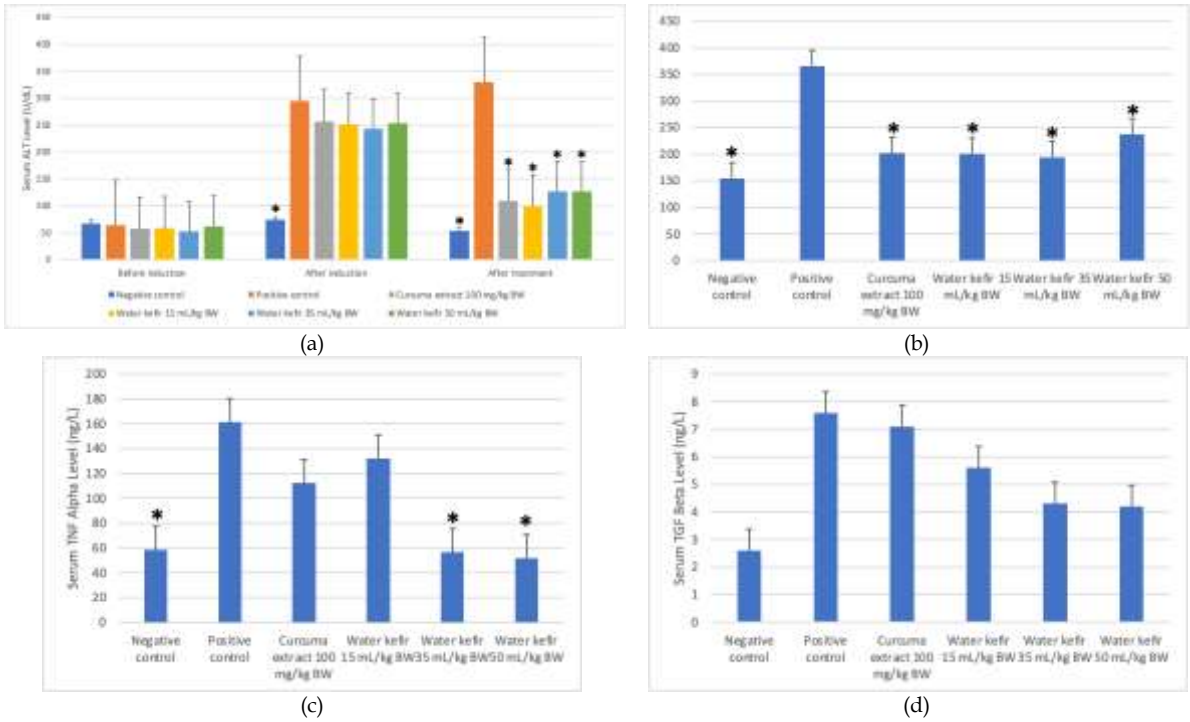


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF-α level; (d) After treatment serum TGF-β level. * indicates a significantly different result when compared to the positive control group; # indicates a significantly different result when compared to the curcuma extract group; $p < 0.05$; n = 4 mice in each group.

A histological examination of a normal liver group revealed a typical central vein bordered with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl4-induced group developed centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The group that received either curcuma extract or water kefir treatment improved in varied necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir 30 mL/kg BW (Fig 2I & 2J)).

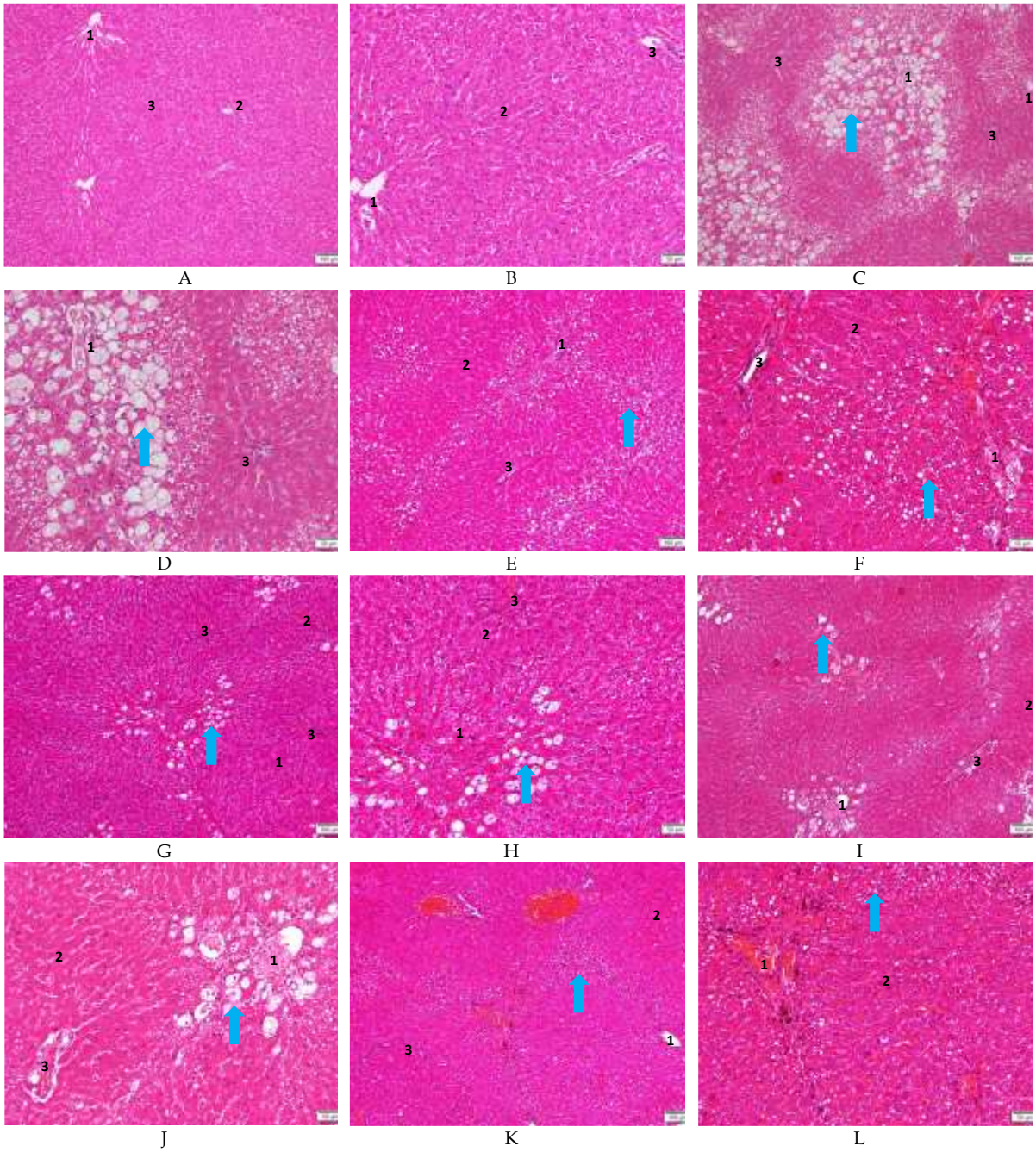
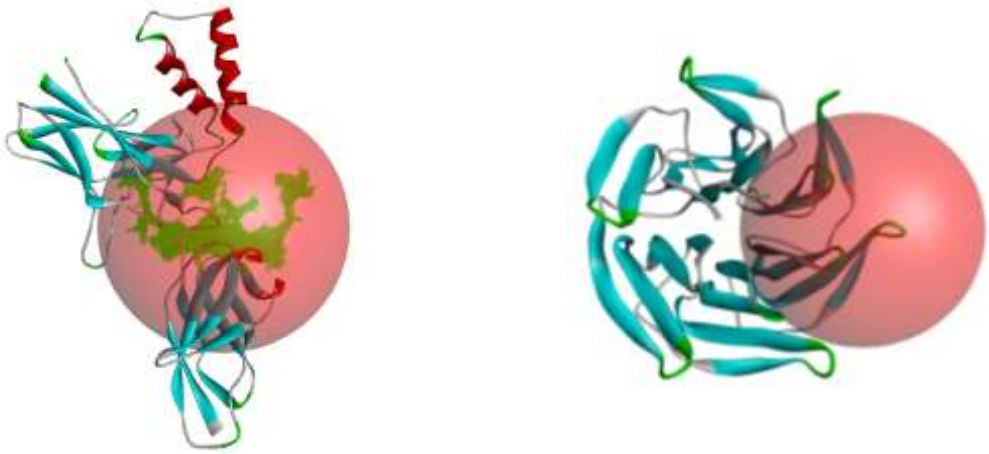


Fig. 2. Liver histology after CCl4 intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal liver epithelial cell; 3: portal track; blue arrow: necrosis and liver epithelial cell vacuolization at centrolobular region.

201

202 **Molecular docking**

203 Molecular docking studies are considered a powerful tool for predicting the potential
204 targets of bioactive molecules. In order to carry out molecular docking simulations, one of the
205 most critical steps is to identify the target active site. If the target protein is crystallized with a
206 native ligand, in many instances, the location of the active site can be established without any
207 difficulty (Li et al. 2019b). However, the NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B)
208 proteins do not have a native ligand, so the active site was determined. Active site prediction
209 in docking is a computational method for predicting the location and orientation of a receptor
210 protein's binding site for a ligand molecule. The active site prediction was based on a protein
211 structural analysis and the identification of amino acid residues that are likely to interact with
212 the ligand. The projected binding site is then utilized as a starting point for molecular docking,
213 a computer method for predicting a ligand molecule's binding affinity and orientation to a
214 receptor protein. The active site prediction for target proteins (Keap1 and NF-κB) gives the
215 grid box coordinates (x y z) of 17.500880 Å, 62.323000 Å, and 0.973748 Å; and -1.751769 Å, -
216 20.743853 Å, and -29.010438 Å, respectively (Fig. 3).



A B

Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF-κB (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

217

218 The docking results of the 25 metabolites could interact with target proteins (Keap1 and
219 NF-κB) (Table 1). In general, all metabolites could interact with both NF-κB receptors (PDB ID
220 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). The interaction of metabolites with nuclear
221 factor-kappa B receptors (PDB ID 1A3Q) revealed that 13 metabolites possessed free energy
222 for binding that was greater than that of curcumin. On the other hand, there was not a single
223 metabolite that had a free energy binding that was more powerful than that of nrf2 Keap1
224 (PDB ID 4L7B) (Table 1).

225 For volatile compounds, 2-phenyl ethanol and benzaldehyde interact most strongly with
226 the NF-κB receptor (PDB ID 1A3Q) with almost the same binding energy of -4.19 kcal/mol.
227 As for organic acids, succinic acid, fumaric acid, and citric acid provide nearly the same strong
228 interactions. Bond energy values of fumaric acid, succinic acid, and citric acid of -6.66, -6.24,
229 and -6.25 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the
230 NF-κB receptor (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2
231 Keap1 receptor (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to
232 interact most strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46
233 kcal/mol. As for organic acids, succinic acid provides nearly the same strong interaction with

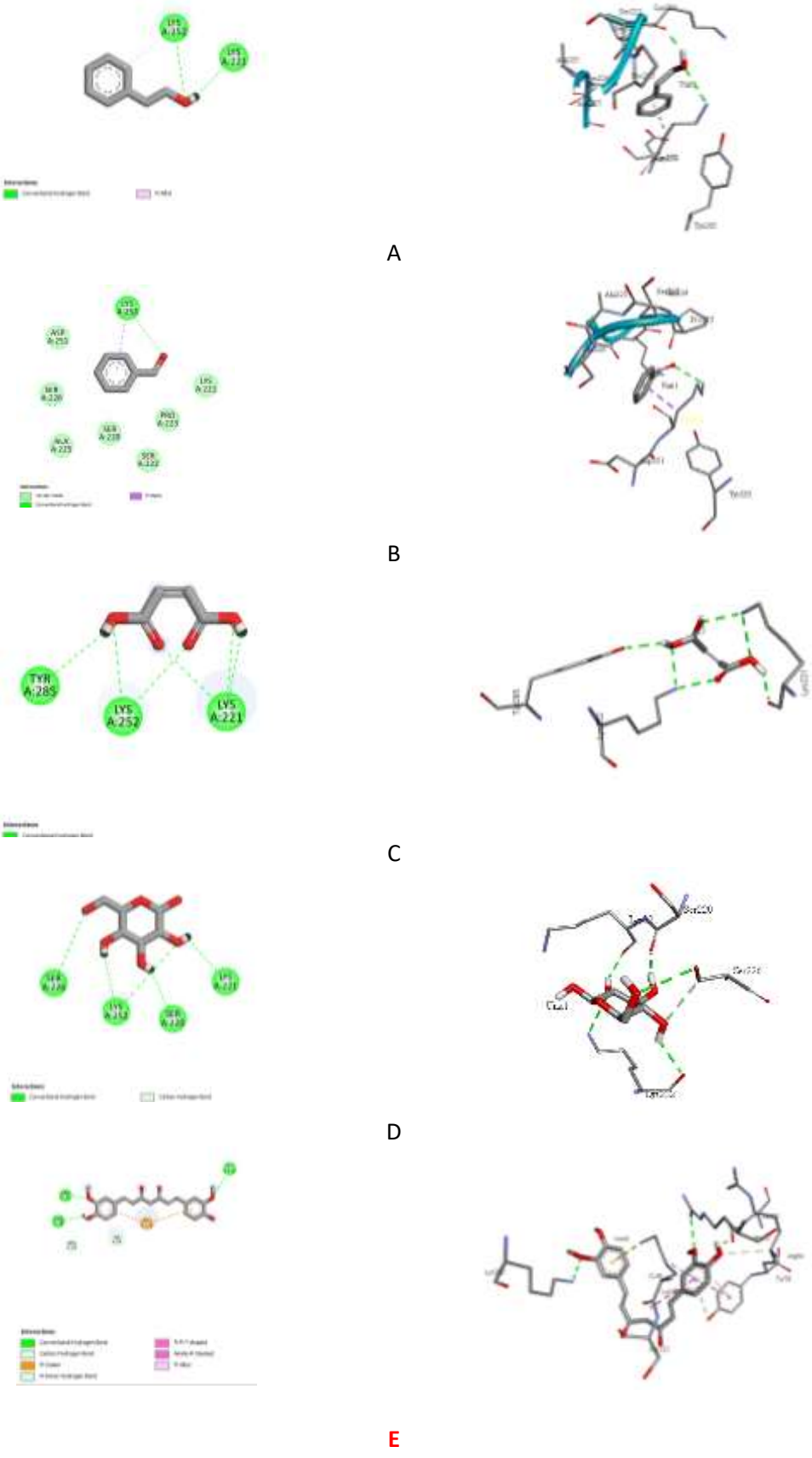
a bond energy value of -3.02 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-κB (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B)

No.	Metabolites	PDB: 1A3Q Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)	PDB: 4L7B Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)
	Volatile compounds				
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-4.19	851.52	-3.12	5,140
4	Ethyl acetate	-3.19	4,590	-2.43	16,670
5	2-Methylbutanal	-3.26	4,050	-2.35	19,050
6	1-Octanol	-2.65	11,490	-2.11	28,220
7	2-Phenylethyl acetate	-3.99	1,190	-3.33	3,590
8	Benzyl acetate	-4.16	887.66	-3.44	3,020
9	Benzaldehyde	-4.19	853.74	-3.19	4,550
10	Furfuryl acetate	-3.60	2,320	-3.32	3,660
11	Isobutyl acetate	-3.19	4,610	-2.94	7,020
12	Isopentyl acetate	-3.20	4,510	-3.05	5,820
13	2-Phenylacetaldehyde	-4.07	1,030	-3.46	2,930
14	3-Methylbutanal	-3.16	4,850	-2.73	10,020
15	Gluconic acid	-3.11	5,280	-1.63	64,290
	Organic acids				
16	Lactic acid	-4.92	249.28	-2.06	30,680
17	Succinic acid	-6.24	26.68	-3.02	6,140
18	Fumaric acid	-6.66	13.22	-2.45	16,100
19	Citric acid	-6.25	26.18	-1.87	42,440
20	Malic acid	-5.65	72.69	-2.53	13,860
21	Acetic acid	-4.99	219.33	-2.44	16,280
22	Ethanol	-2.39	17,800	-2.08	29,800
	Sugars				
23	Sucrose	-1.40	93,710	-1.33	105,320
24	Glucose	-3.71	1,900	-3.09	5,440
25	Fructose	-3.21	4,400	-2.65	11,440
	Reference compound				
	Curcumin	-3.44	2,990	-4.22	811.36

The theoretical binding modes of the top three metabolites with their target proteins (Keap1 and NF-κB) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested that these metabolites interacted with the Keap1 and NF-κB to form a complex through hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site of NF-κB was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid residues of PRO223, LYS252, and LYS252, respectively. The interaction of benzaldehyde with the active site of NF-κB was formed by a hydrogen bond with the amino acid residue of LYS252. The interaction of fumaric acid with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction of glucose with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were able to be formed by six hydrogen bonds, different types of amino acid residues were involved

in the interaction, so fumaric acid interacted more strongly with the active site of NF-κB (Fig. 4).



A

B

C

D

E

Insert here the figures corresponding to curcumin.

Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, D) Glucose, and E) Curcumin.

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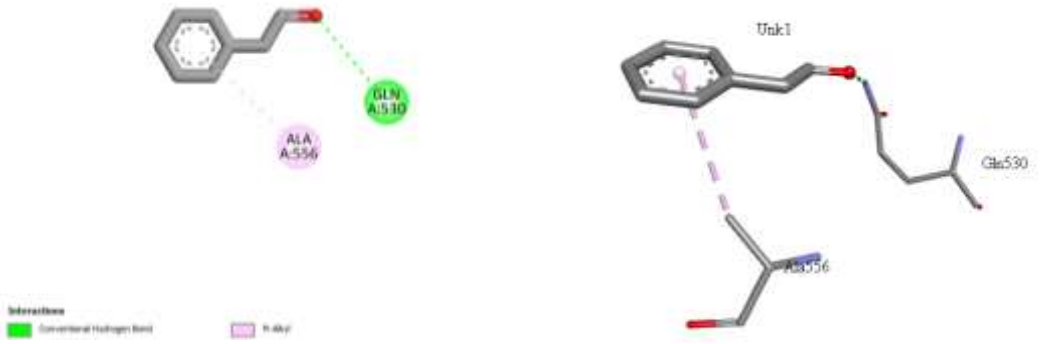
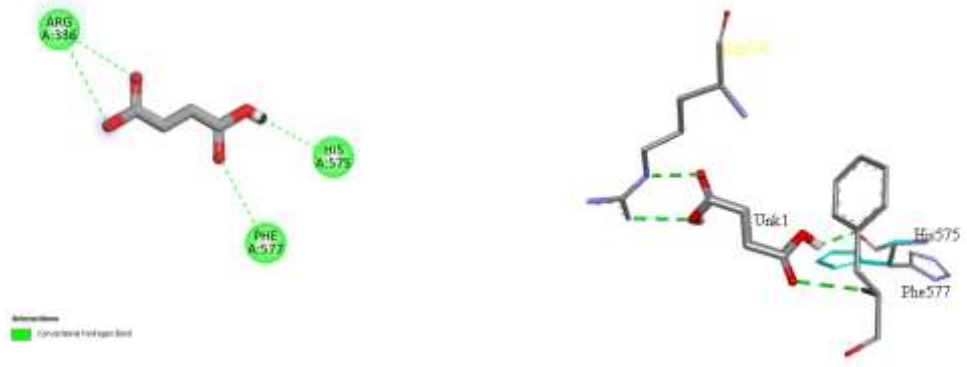


A



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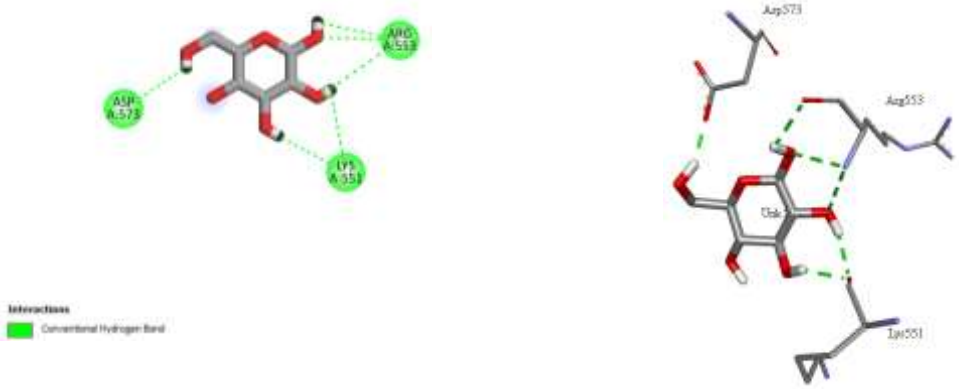

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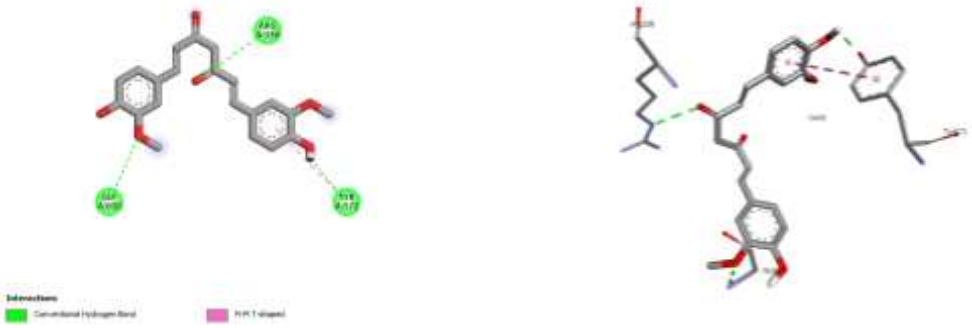

D

Insert here the figures corresponding to curcumin.

Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B). A) 2-Phenylacetaldehyde, B) Succinic acid, C) Glucose, and D) Curcumin.

The interaction of 2-phenylacetaldehyde with the active site of Keap1 was formed by one hydrogen bond and one Pi-Alkyl interaction with amino acid residues of GLN530 and ALA556, respectively. The interaction of succinic acid with the active site of Keap1 was formed by four hydrogen bonds with the amino acid residues of ARG336, HIS575, and PHE577. The interaction of glucose with the active site of Keap1 was formed by six hydrogen bonds with the amino acid residues LYS551, ARG553, and ASP573. Although glucose can form more hydrogen bonds than 2-phenylacetaldehyde, the different types of amino acid residues involved have not been able to have a significant effect on the binding energy of its interaction with the active site of Keap1 (Fig. 5).

Curcumin (the reference compound) created three hydrogen bonds with the amino acid residues ARG52, GLU58, and LYS252 to interact with the active site of NF-κB. Meanwhile, curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572, and GLY600 to interact with the active site of nrf2 Keap1 (Figs. 4 and 5). Despite the fact that curcumin could create three hydrogen bonds at both the active sites of NF-κB and nrf2 Keap1, its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol, benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to interact with NF-κB and nrf2 Keap1 receptors. Whereas fumaric acid and 2-phenylacetaldehyde were metabolites that had the strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

DISCUSSION

Increased liver enzyme production is one of the abnormalities indicating liver damage. This increase in secretion increases the leakage of enzymes into the circulation. Transaminase is a frequent enzyme used to evaluate liver disorders. The transaminases AST and ALT catalyze the transfer of the α-amino group from alanine and aspartic acid to α-ketoglutaric acid. AST is found in the liver, heart, and skeletal muscle, as well as the kidney, brain, pancreas, lung, leukocytes, and erythrocytes. While ALT is predominantly found in the liver, it is also found in low concentrations in other tissues (Lee et al. 2012). Consequently, ALT was used as the principal hepatotoxicity criterion in this study.

Carbon tetrachloride (CCl_4) is a component of the hepatotoxin, which acts after metabolic activation and is extensively employed as a liver-damaging agent. In this study, the administration of CCl_4 to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administrations. CCl_4 is metabolized by the enzyme cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl peroxyl radical, which rapidly reacts with lipids to form lipid peroxidation products. Free radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased protein synthesis, and increased oxidase activity. CCl_4 hepatotoxicity is characterized by hepatocellular necrosis with fat deposition (Ritesh et al. 2015). At the molecular level, administration of CCl_4 can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (Weber et al., 2003).

In terms of its pathophysiological underpinnings, liver illness is linked to a condition known as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Zavala et al., 2016; Laureys and De Vuyst, 2017; Romero-Luna et al., 2020). Both qualitative and quantitative changes in the gut microbiome have the potential to affect the composition of products produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et al., 2020). Qualitative changes include an imbalance between harmful and helpful microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of microbial products can all be caused by dysbiosis (Laureys et al., 2018). For this reason, the condition of the gut microbiome is an important factor in the initiation and development of chronic liver disease (Lee et al., 2021). Based on the results of the study, treatment with water kefir for 2 weeks after the occurrence of liver damage was able to improve the overall condition of the liver, which was marked by a significant decrease in the values of AST, ALT, TNF-α, TGF-β, and significant improvement in liver histology.

Water kefir contains a number of microorganisms that have been linked to health benefits, such as the probiotics *L. paracasei* and *B. cereus* (Fijan 2014). This activity is linked to an increase in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4). Improvements in intestinal barrier function and histological integrity were also observed. Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs simultaneously with the restoration of the p38 MAPK pathway (Fijan 2014; Yao et al. 2019; Tsai et al. 2020; Ji et al. 2022). Bacillus is a kind of endospore-forming bacterium that can endure extremely cold temperatures and lengthy periods of storage without dying; its spores can even tolerate the acidic environment of the stomach and make it all the way to the small intestine (Elshaghabee et al. 2017). *B. cereus* has been shown to reduce ALT levels, an indicator of liver healing, in various animal models of liver injury. It protects the liver by reducing inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines (Kim et al. 2018; Li et al. 2019a; Xue et al. 2020). Also, when *Bacillus* spores were used first, hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al. 2020).

Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria apart from others is their alkaline-stable lipid membrane (Lynch et al. 2021). Their "oxidative" fermentation metabolism is responsible for the principal metabolic process in these bacteria, the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond, fermentation helps mediate the transition of diverse substrates into products. Although lactic acid bacteria have been studied more extensively than acetic acid bacteria (Semjonovs et al. 2014; Hong et al. 2021), various studies have shown promising results concerning the pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the severity of liver injury in rats with septic shock by increasing the expression of TRIM40. TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of cytokines such as IL-6 and TNF-α, raise the expression of IL-10, improve survival in septic mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased inflammation as well as the production of inflammatory cytokines (Yang et al. 2019). Acetic acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation and reduced hepatic lipid and body fat deposition (Kondo et al. 2009; Li et al. 2018).

Apart from microorganisms that directly provide hepatoprotective effects, the metabolites produced from these microorganisms also have the potential to be hepatoprotective. Molecular docking is a technique that is utilized in the context of NF-κB and Nrf2 to make predictions regarding the binding affinity and orientation of small-molecule inhibitors to their active sites. The transcription factor known as NF-κB is an essential component in the management of both the immune system and the inflammatory response (Dai et al., 2021b). The expression of important inflammatory genes can be inhibited by small-molecule inhibitors that impair the interaction between NF-κB and DNA. These inhibitors have the potential to be used in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (Zhao et al., 2017a; Jiang et al., 2019). Small-molecule inhibitors that disrupt the link between Keap1 and Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be disrupted by small-molecule inhibitors (Zhao et al., 2017b).

Binding energy and Ki are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor protein. Binding energy is the energy released when a ligand binds to a receptor protein, and it is calculated as the difference between the energy of the bound complex and the energy of the unbound ligand and protein (Meng et al., 2011). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites. Both binding energy and Ki are used to predict the binding affinity and selectivity of a ligand to a receptor protein (Du et al., 2016). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites (Du et al., 2016).

Based on the results of an in silico study of water kefir metabolite compounds, it was known that fumaric acid and 2-phenylacetaldehyde have the strongest interactions with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively. Fumaric acid has been studied for its potential as a hepatoprotective agent. Fumaric acid protects rat livers against cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic injury against cadmium in rats (Kaur et al., 2020). Fumaric acid esters were found to ameliorate inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic fat accumulation (Šilhavý et al. 2014).

NF-κB and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF-κB is involved in the inflammatory response, and Nrf2 is involved in the antioxidant response (Ganesh Yerra et al. 2013; Wang et al. 2022a). Both transcription factors have been investigated as potential targets for the development of hepatoprotective agents (Rahman et al. 2021; Gao et al. 2022; Wang et al. 2022a; Li et al. 2023). There is evidence of crosstalk between the Nrf2 and NF-kB pathways (Ganesh Yerra et al. 2013; Gao et al. 2022). The Nrf2 pathway inhibits the activation of the NF-κB pathway by increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and detoxifies (Ganesh Yerra et al. 2013). The crosstalk between Nrf2 and NF-κB could be a new therapeutic target against hepatotoxicity (Gao et al. 2022). Researchers have tried to identify molecule activators of Nrf2 as chemoprevention for ROS-dependent carcinogenesis, while others have focused on identifying Nrf2 inhibitors to increase the sensitivity of cancer cells to chemotherapy (Sharifi-Rad et al., 2023). While NF-κB and Nrf2 are involved in different cellular processes, they have both been investigated as potential targets for the development of hepatoprotective agents. Molecular docking studies have been used to investigate the

interaction of potential hepatoprotective agents with these transcription factors. There is also evidence of crosstalk between the Nrf2 and NF-κB pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF-κB plays a crucial role in regulating inflammation and cell death (Luedde and Schwabe 2011). In response to many stimuli that may pose a threat to the host, NF-κB is activated, setting in motion processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde and Schwabe 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs) include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory cytokines, including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe 2011). When NF-B is activated, a lot of genes with B-binding sites are transcribed. These genes play important roles in controlling inflammation, the immune response, and cell survival. In an NF-κB-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by reducing BAMBI expression (an inhibitory TGF-β pseudoreceptor) and increasing Kupffer cell chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF-β, which stimulates HSCs unrestrictively. When HSCs have been activated, NF-κB serves a second crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine way, all play roles in activating NF-κB in activated hepatic stellate cells. More activated HSCs and extracellular matrix are deposited in the liver as a result of greater HSC activation and survival (Luedde and Schwabe 2011).

TNF-α and TGF-β can stimulate one another's production, and TNF-α has been shown to influence TGF-β expression in a variety of cells and tissues (Liu et al. 2022). TNF-α is an inflammatory cytokine that contributes to liver inflammation, and chronic liver inflammation results in liver fibrosis. TNF-α exerts its effects on liver fibrosis via multiple mechanisms, including TGF-β signaling activation (Yang and Seki 2015). Targeting TNF-α and TGF-β signaling pathways may, therefore, have therapeutic potential for treating liver diseases. In regards to hepatoprotective effects, the relationship between TGF-β and TNF-α is complex and not completely understood.

CONCLUSION

This study evaluated the hepatoprotective qualities of Indonesian water kefir in rats with CCl4-induced liver damage. Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF, TGF, and an improvement of liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Hepatoprotective study of Indonesian water kefir against CCl_4-induced liver injury in rats

Running title: Hepatoprotective study of water kefir

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Design	X	X	X	X
Definition of intellectual content	X	X	X	X
Literature search	X			X
Experimental studies	X			X
Data acquisition	X			X
Data analysis	X			X
Statistical analysis	X			X
Manuscript preparation	X	X	X	X
Manuscript editing	X	X	X	X
Manuscript review	X	X	X	X

ABSTRACT

Context: Water kefir is a fermented beverage that is typically made in the home by inoculating a sugar-rich solution with a microbial community (water kefir grains). Several studies on the metabolite content and hepatoprotective effects of water kefir have been published, but CCl_4-induced acute liver injury has not been studied.

Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl_4-induced acute liver injury and to in silico investigate metabolites that play an important role in hepatoprotective mechanisms.

Methods: The present study aimed to investigate the hepatoprotective activity of water kefir in an animal model caused by carbon tetrachloride (CCl4). Furthermore, using molecular docking, the metabolites found in water kefir were evaluated for their role in the NF-κB and Nrf2 signaling pathways.

Results: Water kefir significantly and dose-dependently alleviated acute liver injury caused by carbon tetrachloride (CCl4). Water kefir administration at all doses produced results comparable to the positive control (Curcuma extract). Molecular docking simulations showed that, compared to Nrf2, the 25 metabolites were more likely to interact with the NF-B receptor. Fumaric acid is the strong metabolite that interacts with the NF-κB receptor with a free energy of binding and an inhibition constant of -6.66 kcal/mol and 13.22 μM, respectively.

Conclusions: Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF-α, TGF-β, and an improvement in the liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

Keywords: free radicals; liver diseases; kefir; molecular docking simulation; probiotics

INTRODUCTION

In most cases, making water kefir involves combining dried fruit, sugar, and water kefir grains in a container. Water kefir's exact origins are unknown; however, two hypotheses have been proposed regarding its history: the first suggests that water kefir grains were brought to Europe from the Caucasus by soldiers returning from the Crimean War in the late nineteenth century (Ward, 1892); the second theory proposes that water kefir grains originated in Mexico from the *Opuntia cactus* through natural processes (Moinas et al., 1980). Sugary kefir grains, Balm of Gilead, African bees, Japanese beer seeds, Ale nuts, and California bees are some other names for water kefir. Tibi grains and ginger beer plants are other names for water kefir (Kebler, 1921; Moinas et al., 1980). Water kefir is appealing to both consumers and researchers due to the variety of microbiota it contains, the fact that it is an alternative to dairy products, the versatility with which it can be flavored, the fact that it is low in calories and sugar, the ease with which it can be produced, and the health benefits it offers.

Water kefir has been used medicinally for a very long time, and recent research has indicated that it may have a variety of positive effects on people's health. It has been demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*, *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus ochraceus, A. niger, A. flavus, Penicillium sp.*, and *Rhizopus sp*. (Al-Mohammadi et al., 2021). In addition to its antibacterial properties, water kefir possesses a broad spectrum of pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al., 2003; Aligita et al., 2020), antioxidant (Aligita et al., 2020; Darvishzadeh et al., 2021), hepatoprotective (Aspiras et al., 2015; Aligita et al., 2021), antihyperglycemic and antihyperlipidemic (Alsayadi et al., 2014; Rocha-Gomes et al., 2018), anti-edematous (Moreira et al., 2008), antitumor (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019), immunomodulant (Calatayud et al., 2021), and anti-ulcerogenic (Rodrigues et al., 2016). However, no studies have been reported on the hepatoprotective effects of water kefir against carbon tetrachloride (CCl_4)-induced liver injury.

Studies have shown that acute liver injury is frequently accompanied by high levels of oxidative stress and inflammatory responses (Dai et al., 2021). These findings have been found in several studies. The most important signaling pathways that are involved in the regulation of inflammation and antioxidation are the nuclear factor (NF-κB) and nuclear factor erythroid

2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and inhibiting NF-κB can reduce the amount of damage done to the liver. For instance, curcumin protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1) (Wang et al., 2022b). Also, it has been shown that the molecule methoxy eugenol, which comes from nutmeg and Brazilian red propolis, protects the liver both *in vitro* and *in vivo*. This may be attributed to the fact that it targets the NF-κB signaling pathway, which has been shown to have anti-inflammatory effects (De Souza Basso et al., 2021).

Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage. Additionally, a variety of aromatic and volatile compounds are produced, including ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others (compared to their threshold levels) (Laureys and De Vuyst 2014). The chemical constituents of both phytochemicals and secondary metabolites in natural products, including water kefir, are certainly capable of providing various pharmacological effects for the body (Asnawi et al., 2022; Nursamsiar et al., 2022). However, an *in silico* study to evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity to speed up the process of identifying and optimizing lead compounds, the *in silico* method has become the front-runner in the race to improve the speed and accuracy of the process of discovering new drugs. This is because the *in silico* method can identify and optimize lead compounds more quickly. Techniques such as molecular docking and molecular dynamics (MD) were able to directly indicate a small number of compounds that have high affinity and selectivity by analyzing how the ligand and target interact with one another (Febrina et al., 2021).

Water kefir has been used for an extensive period of time and has been recognized for its widespread benefits, especially in Indonesia. However, its level of popularity falls short in comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl_4-induced rats while also investigating the stability interactions of its metabolites within the NF-κB and Nrf2 receptors using molecular docking studies.

MATERIAL AND METHODS

Materials and reagents

Water kefir grains, sugar, dried fruit, carbon tetrachloride, rats, diagnostic kits for alanine aminotransferase (ALT) (Proline, IFCC mod.), aspartate aminotransferase (AST) (Proline, IFCC mod.), Elisa Kit TNF-α (Bioassay Technology Laboratory), Elisa Kit TGF-β (Bioassay Technology Laboratory). Other chemicals used in this study were of analytical reagent grade.

Experimental sample and reference extract

The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution was produced using a fermentation procedure. The initial stage involved the preparation of 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The sugar and warm distilled water were mixed in a beaker, followed by the addition of water kefir grains and raisins to the resulting sugar solution. The fermentation procedure was conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

to cover the beaker glass. The kefir grain was utilized in future production, while the filtrate was employed for the purpose of evaluation and analysis. (Aligita et al., 2020, 2021)

The rhizoma extract of Curcumae (*Curcuma xanthorrhiza* Roxb) is employed as a reference drug. The utilized product is a standardized herbal medicine with the brand name Tulak, manufactured by the Borobudur Company Herbal Medicine Industry. The primary purpose of Tulak capsules is to support and preserve optimal liver functionality.

Animals and experimental design

Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water *ad libitum*. Four mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) with the ethical approval number 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats received CCl_4 (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al. 2011). The rats were randomized into five groups after the development of animals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with the addition (6) negative control group. Each group received group-specific treatment for two weeks, along with the administration of 1.25 mL/kg BW of CCl_4 (20% in olive oil) every three days.

The rats, which had undergone a fasting period of 8–10 hours while being provided with water, had their blood samples collected via the retro-orbital sinus using a hematocrit capillary tube. The blood sample was taken into a microtube and afterwards then to centrifugation. The serum was separated in order to facilitate further measurements (Parasuraman et al., 2010). Serum ALT level, as the main parameter, was measured prior to induction, following induction, and following treatment. Meanwhile, following therapy, serum AST, TNF-α, TGF-β levels, and liver histopathological were evaluated. The ALT, AST, TNF-α, TGF-β levels measurements are conducted in accordance with the protocols outlined in the reagent kit.

After the euthanasia procedure, the liver specimen was promptly immersed in a 10% formalin solution at room temperature for a duration of 24 hours. Subsequently, the tissues were immersed in paraffin, subjected to sectioning, and affixed onto glass microscope slides. The slices underwent staining with hematoxylin and eosin and were afterwards analyzed using light microscopy (Konstantopoulos et al., 2017).

Molecular docking simulation

Molecular docking experiments were done with the PyRx software (Dallakyan and Olson, 2015) to predict how metabolites, which are small-molecule ligands, bind to biological macromolecules. The NCBI PubChem database (https://pubchem.nlm.nih.gov/, accessed on 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites (Patel et al., 2022). Target proteins like NF-κB (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/, accessed on 03 May 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et al., 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study (Febrina et al., 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the protein and ligand complex and distance (Ischak et al., 2023). The BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

Statistical analysis

All of the information is displayed in the form of individual data points as well as the mean along with the standard error of the mean. The statistical analysis was carried out with the help of Minitab software (version 19.0), and to make comparisons between several different groups, a one-way analysis of variance with Dunnett's post hoc test was utilized. All statistical graphs were created with Microsoft Excel 2019 in their respective versions. The levels of significance that were considered to have been reached were *$p < 0.05$.

RESULTS

In vivo evaluation of hepatoprotective activity

The serum ALT levels, as the main parameter for the liver damage, were measured prior to and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment AST, TNF-α, and TGF-β levels were also evaluated. These findings were analyzed using a one-way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant rise in blood ALT levels demonstrated the establishment of an animal model with liver injury, according to statistical analysis, following the administration of CCl_4. When compared to the positive control group, ALT serum levels decreased significantly after two weeks of therapy with curcuma extract or water kefir. The three doses of water kefir groups demonstrated equivalent activity when curcuma extract was used as the standard treatment, and there was no significant difference between the three doses of water kefir. When compared to the positive control group, AST levels were also reduced dramatically following treatment with curcuma extract or water kefir. TNF-α levels in the water kefir group were significantly lower than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though there was no statistically significant difference in TGF-β levels, the group that received the treatment demonstrated a decrease in TGF-β levels.

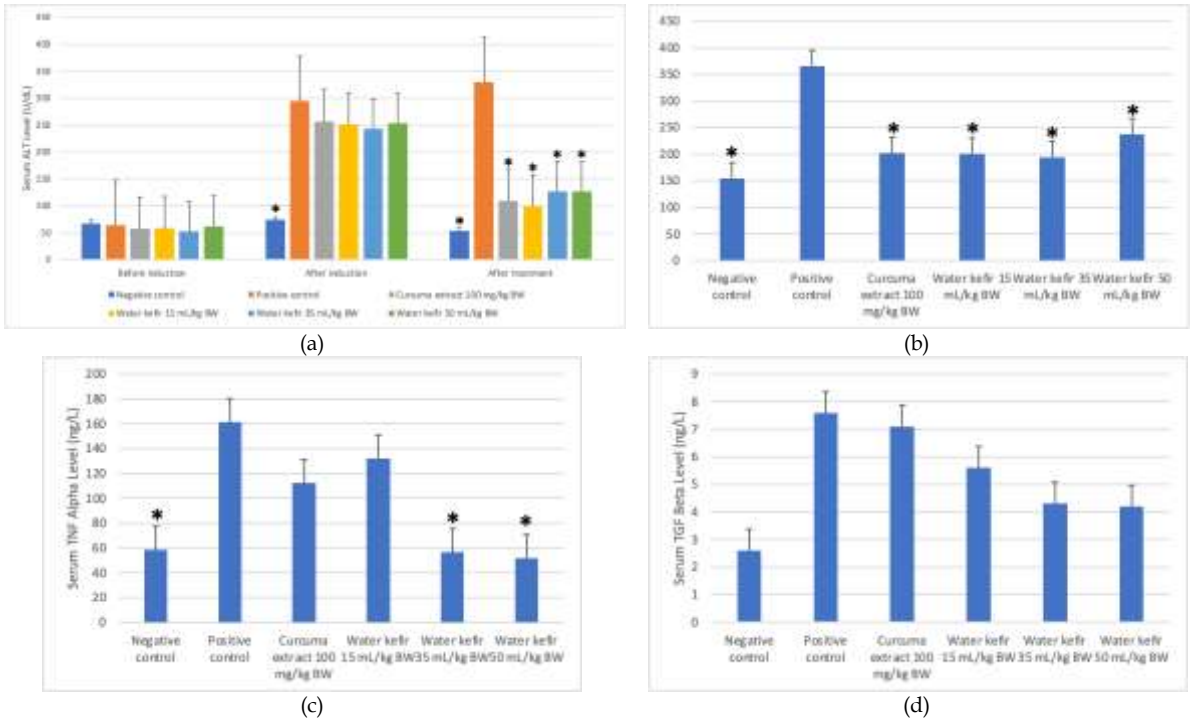


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF-α level; (d) After treatment serum TGF-β level. * indicates a significantly different result when compared to the positive control group; # indicates a significantly different result when compared to the curcuma extract group; $p < 0.05$; n = 4 mice in each group.

A histological examination of a normal liver group revealed a typical central vein bordered with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl4-induced group developed centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The group that received either curcuma extract or water kefir treatment improved in varied necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir 30 mL/kg BW (Fig 2I & 2J)).

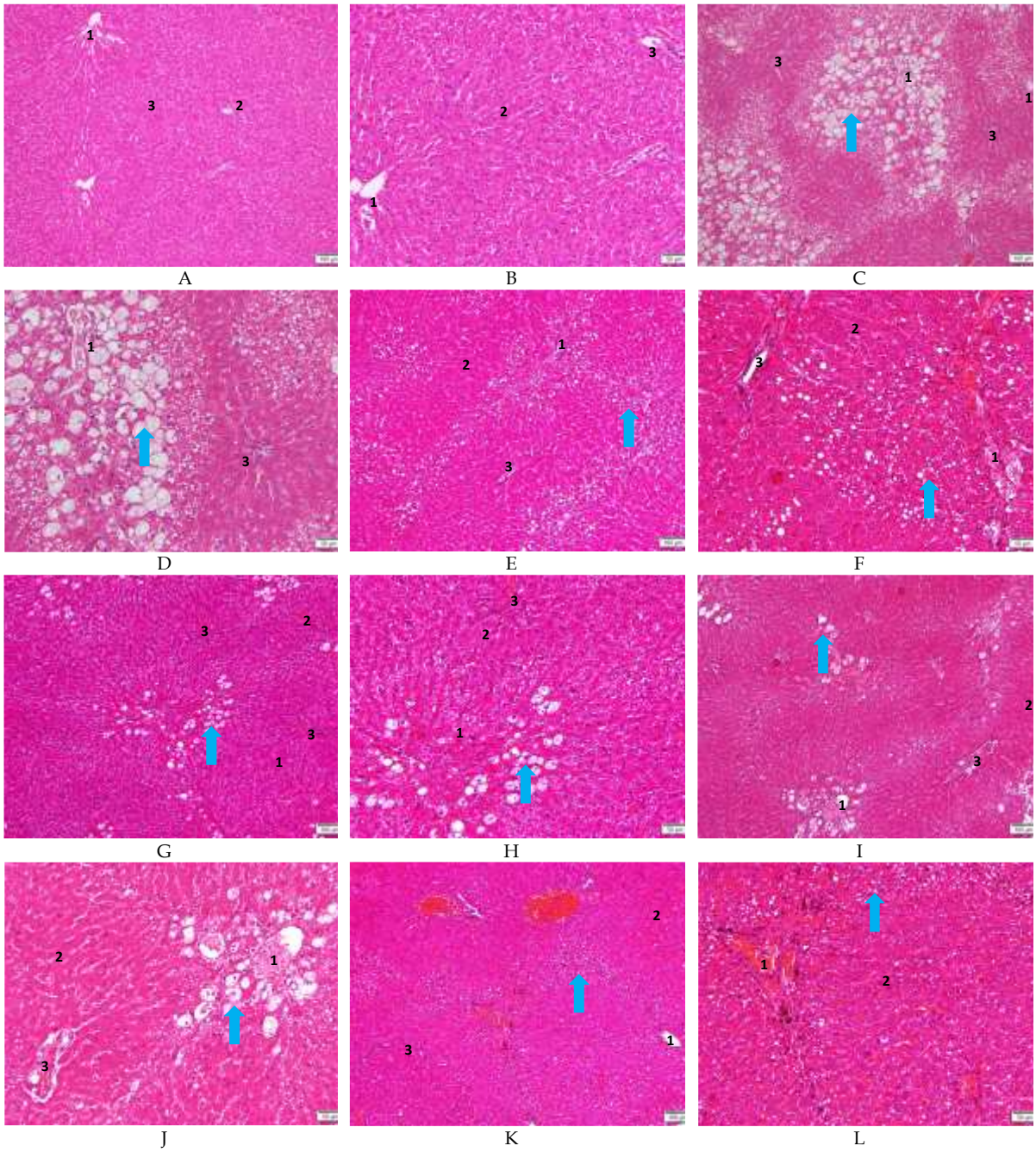
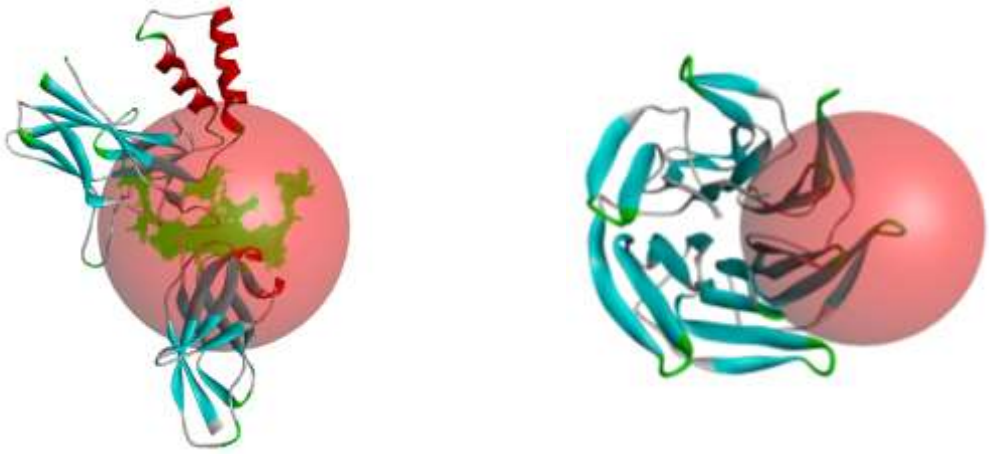


Fig. 2. Liver histology after CCl4 intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal liver epithelial cell; 3: portal track; blue arrow: necrosis and liver epithelial cell vacuolization at centrolobular region.

Molecular docking

Molecular docking studies are considered a powerful tool for predicting the potential targets of bioactive molecules. In order to carry out molecular docking simulations, one of the most critical steps is to identify the target active site. If the target protein is crystallized with a native ligand, in many instances, the location of the active site can be established without any difficulty (Li et al., 2019b). However, the NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) proteins do not have a native ligand, so the active site was determined. Active site prediction in docking is a computational method for predicting the location and orientation of a receptor protein's binding site for a ligand molecule. The active site prediction was based on a protein structural analysis and the identification of amino acid residues that are likely to interact with the ligand. The projected binding site is then utilized as a starting point for molecular docking, a computer method for predicting a ligand molecule's binding affinity and orientation to a receptor protein. The active site prediction for target proteins (Keap1 and NF-κB) gives the grid box coordinates (x y z) of 17.500880 Å, 62.323000 Å, and 0.973748 Å; and -1.751769 Å, -20.743853 Å, and -29.010438 Å, respectively (Fig. 3).



A B

Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF-κB (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

The docking results of the 25 metabolites could interact with target proteins (Keap1 and NF-κB) (Table 1). In general, all metabolites could interact with both NF-κB receptors (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). The interaction of metabolites with nuclear factor-kappa B receptors (PDB ID 1A3Q) revealed that 13 metabolites possessed free energy for binding that was greater than that of curcumin. On the other hand, there was not a single metabolite that had a free energy binding that was more powerful than that of nrf2 Keap1 (PDB ID 4L7B) (Table 1).

For volatile compounds, 2-phenyl ethanol and benzaldehyde interact most strongly with the NF-κB receptor (PDB ID 1A3Q) with almost the same binding energy of -4.19 kcal/mol. As for organic acids, succinic acid, fumaric acid, and citric acid provide nearly the same strong interactions. Bond energy values of fumaric acid, succinic acid, and citric acid of -6.66, -6.24, and -6.25 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the NF-κB receptor (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2

Keap1 receptor (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to interact most strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46 kcal/mol. As for organic acids, succinic acid provides nearly the same strong interaction with a bond energy value of -3.02 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-κB (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B)

No.	Metabolites	PDB: 1A3Q Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)	PDB: 4L7B Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μM)
	Volatile compounds				
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-4.19	851.52	-3.12	5,140
4	Ethyl acetate	-3.19	4,590	-2.43	16,670
5	2-Methylbutanal	-3.26	4,050	-2.35	19,050
6	1-Octanol	-2.65	11,490	-2.11	28,220
7	2-Phenylethyl acetate	-3.99	1,190	-3.33	3,590
8	Benzyl acetate	-4.16	887.66	-3.44	3,020
9	Benzaldehyde	-4.19	853.74	-3.19	4,550
10	Furfuryl acetate	-3.60	2,320	-3.32	3,660
11	Isobutyl acetate	-3.19	4,610	-2.94	7,020
12	Isopentyl acetate	-3.20	4,510	-3.05	5,820
13	2-Phenylacetaldehyde	-4.07	1,030	-3.46	2,930
14	3-Methylbutanal	-3.16	4,850	-2.73	10,020
15	Gluconic acid	-3.11	5,280	-1.63	64,290
	Organic acids				
16	Lactic acid	-4.92	249.28	-2.06	30,680
17	Succinic acid	-6.24	26.68	-3.02	6,140
18	Fumaric acid	-6.66	13.22	-2.45	16,100
19	Citric acid	-6.25	26.18	-1.87	42,440
20	Malic acid	-5.65	72.69	-2.53	13,860
21	Acetic acid	-4.99	219.33	-2.44	16,280
22	Ethanol	-2.39	17,800	-2.08	29,800
	Sugars				
23	Sucrose	-1.40	93,710	-1.33	105,320
24	Glucose	-3.71	1,900	-3.09	5,440
25	Fructose	-3.21	4,400	-2.65	11,440
	Reference compound				
	Curcumin	-3.44	2,990	-4.22	811.36

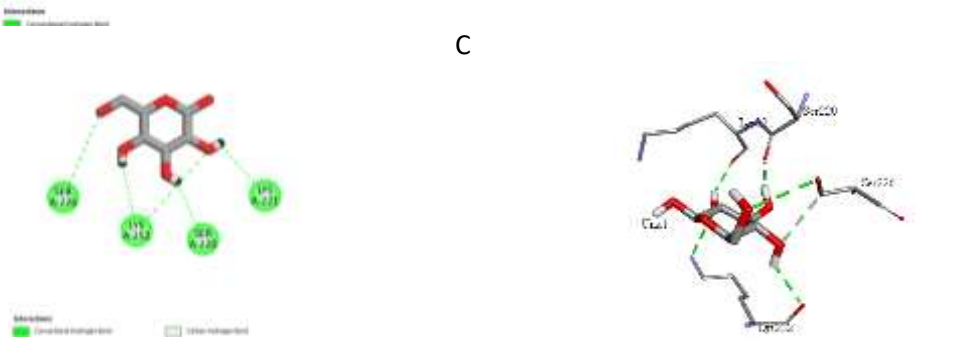
The theoretical binding modes of the top three metabolites with their target proteins (Keap1 and NF-κB) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested that these metabolites interacted with the Keap1 and NF-κB to form a complex through hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site of NF-κB was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid residues of PRO223, LYS252, and LYS252, respectively. The interaction of benzaldehyde with the active site of NF-κB was formed by a hydrogen bond with the amino acid residue of LYS252. The interaction of fumaric acid with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction of glucose with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were able to be formed by six hydrogen bonds, different types of amino acid residues were involved

in the interaction, so fumaric acid interacted more strongly with the active site of NF-κB (Fig. 4).

A

B

C



D

E

Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, D) Glucose, and E) Curcumin.

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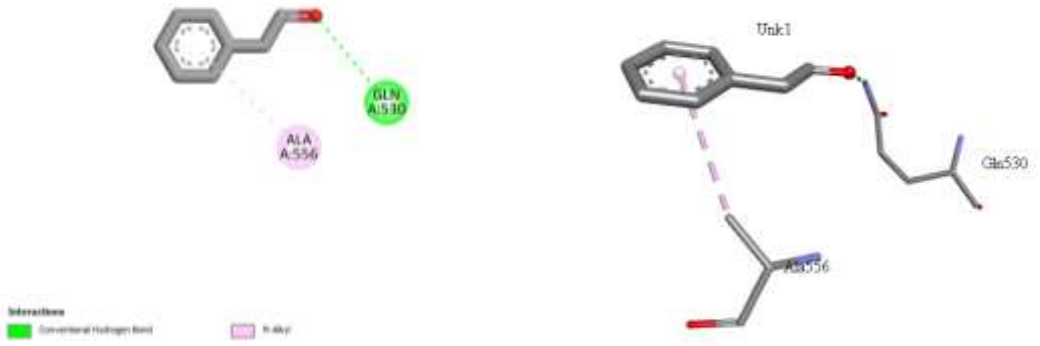
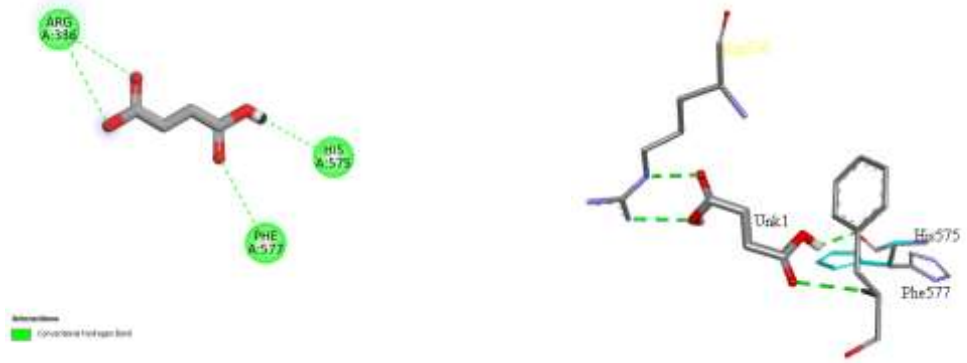
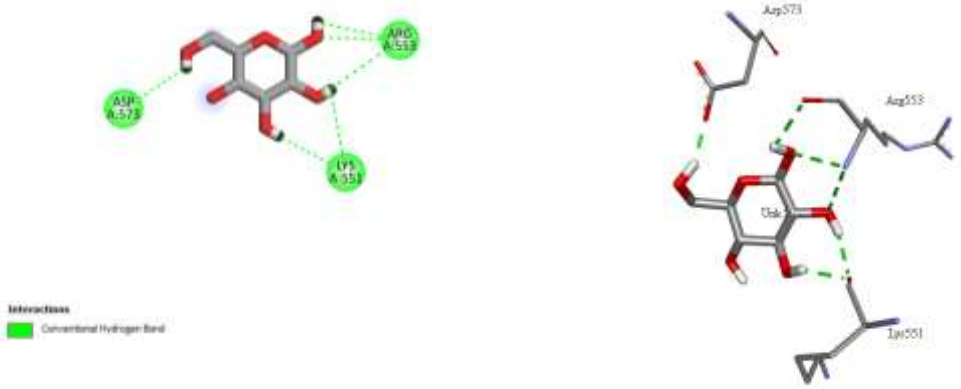
257



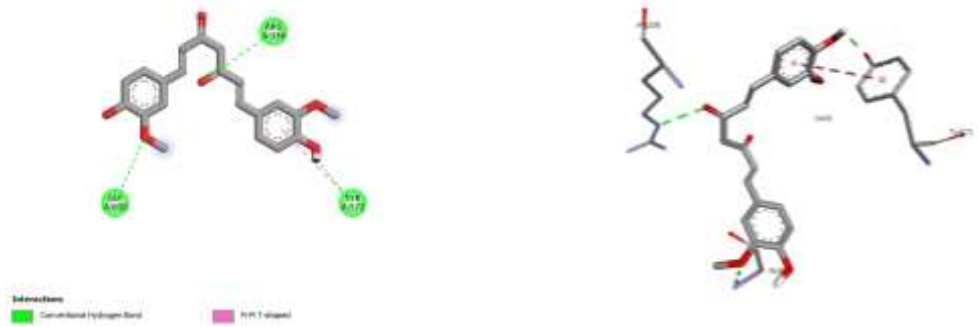
A



B



C



D

Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B). A) 2-Phenylacetaldehyde, B) Succinic acid, C) Glucose, and D) Curcumin.

The interaction of 2-phenylacetaldehyde with the active site of Keap1 was formed by one hydrogen bond and one Pi-Alkyl interaction with amino acid residues of GLN530 and ALA556, respectively. The interaction of succinic acid with the active site of Keap1 was formed by four hydrogen bonds with the amino acid residues of ARG336, HIS575, and PHE577. The interaction of glucose with the active site of Keap1 was formed by six hydrogen bonds with the amino acid residues LYS551, ARG553, and ASP573. Although glucose can form more hydrogen bonds than 2-phenylacetaldehyde, the different types of amino acid residues involved have not been able to have a significant effect on the binding energy of its interaction with the active site of Keap1 (Fig. 5).

Curcumin (the reference compound) created three hydrogen bonds with the amino acid residues ARG52, GLU58, and LYS252 to interact with the active site of NF-κB. Meanwhile, curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572, and GLY600 to interact with the active site of nrf2 Keap1 (Figs. 4 and 5). Despite the fact that curcumin could create three hydrogen bonds at both the active sites of NF-κB and nrf2 Keap1, its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol, benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to interact with NF-κB and nrf2 Keap1 receptors. Whereas fumaric acid and 2-phenylacetaldehyde were metabolites that had the strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

DISCUSSION

Increased liver enzyme production is one of the abnormalities indicating liver damage. This increase in secretion increases the leakage of enzymes into the circulation. Transaminase is a frequent enzyme used to evaluate liver disorders. The transaminases AST and ALT catalyze the transfer of the α-amino group from alanine and aspartic acid to α-ketoglutaric acid. AST is found in the liver, heart, and skeletal muscle, as well as the kidney, brain, pancreas, lung, leukocytes, and erythrocytes. While ALT is predominantly found in the liver, it is also found in low concentrations in other tissues (Lee et al., 2012). Consequently, ALT was used as the principal hepatotoxicity criterion in this study.

Carbon tetrachloride (CCl_4) is a component of the hepatotoxin, which acts after metabolic activation and is extensively employed as a liver-damaging agent. In this study, the administration of CCl_4 to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administrations. CCl_4 is metabolized by the enzyme cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl peroxyl radical, which rapidly reacts with lipids to form lipid peroxidation products. Free radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased protein synthesis, and increased oxidase activity. CCl_4 hepatotoxicity is characterized by hepatocellular necrosis with fat deposition (Ritesh et al., 2015). At the molecular level, administration of CCl_4 can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (Weber et al., 2003).

In terms of its pathophysiological underpinnings, liver illness is linked to a condition known as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Zavala et al.,

2016; Laureys and De Vuyst, 2017; Romero-Luna et al., 2020). Both qualitative and quantitative changes in the gut microbiome have the potential to affect the composition of products produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et al., 2020). Qualitative changes include an imbalance between harmful and helpful microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of microbial products can all be caused by dysbiosis (Laureys et al., 2018). For this reason, the condition of the gut microbiome is an important factor in the initiation and development of chronic liver disease (Lee et al., 2021). Based on the results of the study, treatment with water kefir for 2 weeks after the occurrence of liver damage was able to improve the overall condition of the liver, which was marked by a significant decrease in the values of AST, ALT, TNF-α, TGF-β, and significant improvement in liver histology.

Water kefir contains a number of microorganisms that have been linked to health benefits, such as the probiotics *L. paracasei* and *B. cereus* (Fijan, 2014). This activity is linked to an increase in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4). Improvements in intestinal barrier function and histological integrity were also observed. Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs simultaneously with the restoration of the p38 MAPK pathway (Fijan, 2014; Yao et al., 2019; Tsai et al., 2020; Ji et al., 2022). Bacillus is a kind of endospore-forming bacterium that can endure extremely cold temperatures and lengthy periods of storage without dying; its spores can even tolerate the acidic environment of the stomach and make it all the way to the small intestine (Elshaghabee et al., 2017). *Bacillus cereus* has been shown to reduce ALT levels, an indicator of liver healing, in various animal models of liver injury. It protects the liver by reducing inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines (Kim et al., 2018; Li et al., 2019a; Xue et al., 2020). Also, when *Bacillus* spores were used first, hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al., 2020).

Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria apart from others is their alkaline-stable lipid membrane (Lynch et al., 2021). Their "oxidative" fermentation metabolism is responsible for the principal metabolic process in these bacteria, the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond, fermentation helps mediate the transition of diverse substrates into products. Although lactic acid bacteria have been studied more extensively than acetic acid bacteria (Semjonovs et al., 2014; Hong et al., 2021), various studies have shown promising results concerning the pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the severity of liver injury in rats with septic shock by increasing the expression of TRIM40. TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of cytokines such as IL-6 and TNF-α, raise the expression of IL-10, improve survival in septic mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased inflammation as well as the production of inflammatory cytokines (Yang et al., 2019). Acetic acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation and reduced hepatic lipid and body fat deposition (Kondo et al., 2009; Li et al., 2018).

Apart from microorganisms that directly provide hepatoprotective effects, the metabolites produced from these microorganisms also have the potential to be hepatoprotective.

Molecular docking is a technique that is utilized in the context of NF-κB and Nrf2 to make predictions regarding the binding affinity and orientation of small-molecule inhibitors to their active sites. The transcription factor known as NF-κB is an essential component in the management of both the immune system and the inflammatory response (Dai et al., 2021). The expression of important inflammatory genes can be inhibited by small-molecule inhibitors that impair the interaction between NF-κB and DNA. These inhibitors have the potential to be used in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (Zhao et al., 2017; Jiang et al., 2019). Small-molecule inhibitors that disrupt the link between Keap1 and Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be disrupted by small-molecule inhibitors (Zhao et al., 2017).

Binding energy and Ki are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor protein. Binding energy is the energy released when a ligand binds to a receptor protein, and it is calculated as the difference between the energy of the bound complex and the energy of the unbound ligand and protein (Meng et al., 2011). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites. Both binding energy and Ki are used to predict the binding affinity and selectivity of a ligand to a receptor protein (Du et al., 2016). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites (Du et al., 2016).

Based on the results of an in silico study of water kefir metabolite compounds, it was known that fumaric acid and 2-phenylacetaldehyde have the strongest interactions with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively. Fumaric acid has been studied for its potential as a hepatoprotective agent. Fumaric acid protects rat livers against cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic injury against cadmium in rats (Kaur et al., 2020). Fumaric acid esters were found to ameliorate inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic fat accumulation (Šilhavý et al., 2014).

NF-κB and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF-κB is involved in the inflammatory response, and Nrf2 is involved in the antioxidant response (Ganesh Yerra et al., 2013; Wang et al., 2022a). Both transcription factors have been investigated as potential targets for the development of hepatoprotective agents (Rahman et al., 2021; Gao et al., 2022; Wang et al., 2022a; Li et al., 2023). There is evidence of crosstalk between the Nrf2 and NF-kB pathways (Ganesh Yerra et al., 2013; Gao et al., 2022). The Nrf2 pathway inhibits the activation of the NF-κB pathway by increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and detoxifies (Ganesh Yerra et al., 2013). The crosstalk between Nrf2 and NF-κB could be a new therapeutic target against hepatotoxicity (Gao et al., 2022). Researchers have tried to identify molecule activators of Nrf2 as chemoprevention for ROS-dependent carcinogenesis, while others have focused on identifying Nrf2 inhibitors to increase the sensitivity of cancer cells to chemotherapy (Sharifi-Rad et al., 2023). While NF-κB and Nrf2 are involved in different cellular processes, they have both been investigated as potential targets for the development of hepatoprotective agents. Molecular docking studies have been used to investigate the interaction of potential hepatoprotective agents with these transcription factors. There is also

evidence of crosstalk between the Nrf2 and NF-κB pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF-κB plays a crucial role in regulating inflammation and cell death (Luedde and Schwabe, 2011). In response to many stimuli that may pose a threat to the host, NF-κB is activated, setting in motion processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde and Schwabe, 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs) include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory cytokines, including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe, 2011). When NF-B is activated, a lot of genes with B-binding sites are transcribed. These genes play important roles in controlling inflammation, the immune response, and cell survival. In an NF-κB-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by reducing BAMBI expression (an inhibitory TGF-β pseudoreceptor) and increasing Kupffer cell chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF-β, which stimulates HSCs unrestrictively. When HSCs have been activated, NF-κB serves a second crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine way, all play roles in activating NF-κB in activated hepatic stellate cells. More activated HSCs and extracellular matrix are deposited in the liver as a result of greater HSC activation and survival (Luedde and Schwabe, 2011).

TNF-α and TGF-β can stimulate one another's production, and TNF-α has been shown to influence TGF-β expression in a variety of cells and tissues (Liu et al., 2022). TNF-α is an inflammatory cytokine that contributes to liver inflammation, and chronic liver inflammation results in liver fibrosis. TNF-α exerts its effects on liver fibrosis via multiple mechanisms, including TGF-β signaling activation (Yang and Seki, 2015). Targeting TNF-α and TGF-β signaling pathways may, therefore, have therapeutic potential for treating liver diseases. In regards to hepatoprotective effects, the relationship between TGF-β and TNF-α is complex and not completely understood.

CONCLUSION

This study evaluated the hepatoprotective qualities of Indonesian water kefir in rats with CCl4-induced liver damage. Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF, TGF, and an improvement of liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Bandung, September 21, 2023

Dear **Prof. Gabino Garrido**
Editor in Chief
Journal of Pharmacy & Pharmacognosy Research
Garval Editorial Ltda.
Antofagasta
Chile

Thank you for giving us the opportunity to submit a revised draft of our manuscript (ID: JPPRes-23-1732.R2) titled *Hepatoprotective Study of Indonesian Water Kefir Against CCl4-Induced Liver Injury* in *Rats* to *Journal of Pharmacy & Pharmacognosy Research (JPPRes)*. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable feedback on our manuscript. We are grateful to the reviewers for their insightful comments on our paper. We have been able to incorporate changes to reflect most of the suggestions provided by the reviewers. We have highlighted the changes within the manuscript.

Here is a point-by-point response to the reviewers' comments and concerns

	Comment	**Response**
	Please place a comma before the year in all citations throughout the document. See examples of already arranged citations in the Introduction.	We have revised the citations
Fig 4	Insert here the figures corresponding to curcumin.	The image of curcumin has been listed above the letter E
Fig 5	Insert here the figures corresponding to curcumin.	The image of curcumin has been listed above the letter D
References	This section is one of the most important sections of the document. Please pay close attention to it. The style of the References does not meet the requirements of JPPRes. Please write the References according to the Instructions to the Author or the examples: https://jppres.com/jppres/archive/ https://jppres.com/jppres/volume-11-issue-2/ See examples in 1-3 references. Also, all authors (not "et al.") and DOIs must be given. Please check that all references in this section have been cited in the text and vice versa.	We have revised the references referring to the sample.

Thank you, and we look forward to hearing more about our manuscript.

With kind regards,

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Original Article

Hepatoprotective study of Indonesian water kefir against CCl_4-induced liver injury in rats

[Estudio hepatoprotector del kéfir de agua indonesio contra la lesión hepática inducida por CCl_4 en ratas]

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Abstract

Context: Water kefir is a fermented beverage that is typically made in the home by inoculating a sugar-rich solution with a microbial community (water kefir grains). Several studies on the metabolite content and hepatoprotective effects of water kefir have been published, but carbon tetrachloride (CCl_4)-induced acute liver injury has not been studied.

Aims: To evaluate the efficacy of water kefir *in vivo* against hepatoprotective CCl_4-induced acute liver injury and to in silico investigate metabolites that play an important role in hepatoprotective mechanisms.

Methods: The present study aimed to investigate the hepatoprotective activity of water kefir in an animal model caused by CCl_4. Furthermore, using molecular docking, the metabolites found in water kefir were evaluated for their role in the NF-κB and Nrf2 signaling pathways.

Results: Water kefir significantly and dose-dependently alleviated acute liver injury caused by CCl_4. Water kefir administration at all doses produced results comparable to the positive control (*Curcuma* extract). Molecular docking simulations showed that, compared to Nrf2, the 25 metabolites were more likely to interact with the NF-κB receptor. Fumaric acid is the strong metabolite that interacts with the NF-κB receptor with a free energy of binding and an inhibition constant of -6.66 kcal/mol and 13.22 µM, respectively.

Conclusions: Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF-α, TGF-β, and an improvement in the liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

Keywords: free radicals; liver diseases; kefir; molecular docking simulation; probiotics.

Resumen

Contexto: El kéfir de agua es una bebida fermentada que se suele elaborar en casa inoculando una solución rica en azúcar con una comunidad microbiana (granos de kéfir de agua). Se han publicado varios estudios sobre el contenido de metabolitos y los efectos hepatoprotectores del kéfir de agua, pero no se ha estudiado la lesión hepática aguda inducida por tetracloruro de carbono (CCl_4).

Objetivos: Evaluar la eficacia del kéfir de agua *in vivo* contra la lesión hepática aguda inducida por CCl_4 hepatoprotectora e investigar in silico los metabolitos que desempeñan un papel importante en los mecanismos hepatoprotectores.

Métodos: El presente estudio tuvo como objetivo investigar la actividad hepatoprotectora del kéfir de agua en un modelo animal causado por CCl_4. Además, mediante docking molecular, se evaluó el papel de los metabolitos presentes en el kéfir de agua en las vías de señalización NF-κB y Nrf2.

Resultados: El kéfir de agua alivió de forma significativa y dependiente de la dosis la lesión hepática aguda causada por CCl_4. La administración de kéfir de agua a todas las dosis produjo resultados comparables a los del control positivo (extracto de cúrcuma). Las simulaciones de acoplamiento molecular mostraron que, en comparación con el Nrf2, los 25 metabolitos eran más propensos a interactuar con el receptor NF-κB. El ácido fumárico es el metabolito más potente. El ácido fumárico es el metabolito fuerte que interacciona con el receptor NF-κB con una energía libre de unión y una constante de inhibición de -6,66 kcal/mol y 13,22 µM, respectivamente.

Conclusiones: La administración de kéfir de agua mejoró el estado de daño hepático, caracterizado por una disminución de los niveles séricos de AST, ALT, TNF-α, TGF-β, y una mejora del perfil tisular hepático. La evaluación *in silico* mostró que los metabolitos del kéfir de agua eran capaces de interactuar con proteínas diana en las vías NF-κB y Nrf2. Se concluyó que el kéfir de agua mejora el estado del hígado al reducir el nivel de necrosis y fibrosis.

Palabras Clave: enfermedades hepáticas; kéfir; probióticos; radicales libres; simulación de acoplamiento molecular.

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INTRODUCTION

In most cases, making water kefir involves combining dried fruit, sugar, and water kefir grains in a container. Water kefir's exact origins are unknown; however, two hypotheses have been proposed regarding its history: the first suggests that water kefir grains were brought to Europe from the Caucasus by soldiers returning from the Crimean War in the late nineteenth century (Ward, 1892); the second theory proposes that water kefir grains originated in Mexico from the *Opuntia cactus* through natural processes (Moinas et al., 1980). Sugary kefir grains, Balm of Gilead, African bees, Japanese beer seeds, Ale nuts, and California bees are some other names for water kefir. Tibi grains and ginger beer plants are other names for water kefir (Kebler, 1921; Moinas et al., 1980). Water kefir is appealing to both consumers and researchers due to the variety of microbiota it contains, the fact that it is an alternative to dairy products, the versatility with which it can be flavored, the fact that it is low in calories and sugar, the ease with which it can be produced, and the health benefits it offers.

Water kefir has been used medicinally for a very long time, and recent research has indicated that it may have a variety of positive effects on people's health. It has been demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp., Salmonella sp., Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus ochraceus, A. niger, A. flavus, Penicillium sp.*, and *Rhizopus sp*. (Al-Mohammadi et al., 2021). In addition to its antibacterial properties, water kefir possesses a broad spectrum of pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Aligita et al., 2020; Diniz et al., 2003), antioxidant (Aligita et al., 2020; Darvishzadeh et al., 2021), hepatoprotective (Aligita et al., 2021; Aspiras et al., 2015), antihyperglycemic and antihyperlipidemic (Alsayadi et al., 2014; Rocha-Gomes et al., 2018), anti-edematous (Moreira et al., 2008), antitumor (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019), immunomodulant (Calatayud et al., 2021), and anti-ulcerogenic (Rodrigues et al., 2016). However, no studies have been reported on the hepatoprotective effects of water kefir against carbon tetrachloride (CCl_4)-induced liver injury.

Studies have shown that acute liver injury is frequently accompanied by high levels of oxidative stress and inflammatory responses (Dai et al., 2021). These findings have been found in several studies. The most important signaling pathways that are involved in the regulation of inflammation and antioxidation are the nuclear factor (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and inhibiting NF-κB can reduce the amount of damage done to the liver. For instance, curcumin protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1) (Wang et al., 2022b). Also, it has been shown that the molecule methoxy eugenol, which comes from nutmeg and Brazilian red propolis, protects the liver both *in vitro* and *in vivo*. This may be attributed to the fact that it targets the NF-κB signaling pathway, which has been shown to have anti-inflammatory effects (De Souza Basso et al., 2021).

Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage. Additionally, a variety of aromatic and volatile compounds are produced, including ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others (compared to their threshold levels) (Laureys and De Vuyst 2014). The chemical constituents of both phytochemicals and secondary metabolites in natural products, including water kefir, are certainly capable of providing various pharmacological effects for the body (Asnawi et al., 2022a; Nursamsiar et al., 2022). However, an *in silico* study to evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity to speed up the process of identifying and optimizing lead compounds, the *in silico* method has become the front-runner in the race to improve the speed and accuracy of the process of discovering new drugs. This is because the *in silico* method can identify and optimize lead compounds more quickly. Techniques such as molecular docking and molecular dynamics (MD) were able to directly indicate a small number of compounds that have high affinity and selectivity by analyzing how the ligand and target interact with one another (Febrina et al., 2021).

Water kefir has been used for an extensive period of time and has been recognized for its widespread benefits, especially in Indonesia. However, its level of popularity falls short in comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the purpose of this study was to evaluate the hepatopro-

tective effects of water kefir in CCl_4-induced rats while also investigating the stability interactions of its metabolites within the NF-κB and Nrf2 receptors using molecular docking studies.

MATERIAL AND METHODS

Materials and reagents

Water kefir grains, sugar, dried fruit, carbon tetrachloride, rats, diagnostic kits for alanine aminotransferase (ALT) (Proline, IFCC mod.), aspartate aminotransferase (AST) (Proline, IFCC mod.), Elisa Kit TNF-α (Bioassay Technology Laboratory), Elisa Kit TGF-β (Bioassay Technology Laboratory). Other chemicals used in this study were of analytical reagent grade.

Experimental sample and reference extract

The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution was produced using a fermentation procedure. The initial stage involved the preparation of 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of mineral distillated water. The sugar and warm distilled water were mixed in a beaker, followed by the addition of water kefir grains and raisins to the resulting sugar solution. The fermentation procedure was conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used to cover the beaker glass. The kefir grain was utilized in future production, while the filtrate was employed for the purpose of evaluation and analysis. (Aligita et al., 2020; 2021)

The rhizome extract of *Curcuma* (*Curcuma zanthorrhiza* Roxb, (family *Zingiberaceae*) was employed as a reference drug. The utilized product was a standardized herbal medicine with the brand name Tulak, manufactured by the Borobudur Company Herbal Medicine Industry. The primary purpose of Tulak capsules was to support and preserve optimal liver functionality.

Animals and experimental design

Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water *ad libitum*. Four mice in each group were used. All procedures relating to animals and their care conformed to the international guidelines Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) with the ethical approval number 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats received CCl_4 (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al. 2011). The rats were randomized into five groups after the development of animals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) *Curcuma* extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with the addition (6) negative control group. Each group received group-specific treatment for two weeks, along with the administration of 1.25 mL/kg BW of CCl_4 (20% in olive oil) every three days.

The rats, which had undergone a fasting period of 8–10 hours while being provided with water, had their blood samples collected via the retro-orbital sinus using a hematocrit capillary tube. The blood sample was taken into a microtube and afterwards then to centrifugation. The serum was separated in order to facilitate further measurements (Parasuraman et al., 2010). Serum ALT level, as the main parameter, was measured prior to induction, following induction, and following treatment. Meanwhile, following therapy, serum AST, TNF-α, TGF-β levels, and liver histopathological were evaluated. The ALT, AST, TNF-α, TGF-β levels measurements are conducted in accordance with the protocols outlined in the reagent kit.

After the euthanasia procedure, the liver specimen was promptly immersed in a 10% formalin solution at room temperature for a duration of 24 hours. Subsequently, the tissues were immersed in paraffin, subjected to sectioning, and affixed onto glass microscope slides. The slices underwent staining with hematoxylin and eosin and were afterwards analyzed using light microscopy (Konstantopoulos et al., 2017).

Molecular docking simulation

Molecular docking experiments were done with the PyRx software (Dallakyan and Olson, 2015) to predict how metabolites, which are small-molecule ligands, bind to biological macromolecules. The NCBI PubChem database (https://pubchem.nlm.nih.gov/, accessed on 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites (Patel et al., 2022). Target proteins like NF-κB (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/, accessed on 03 May 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et al., 2023), then maximize GRID parameter (Asnawi et al. 2022b) and perform docking study (Febrina et al., 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the protein and ligand complex and distance (Ischak et al., 2023). The BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

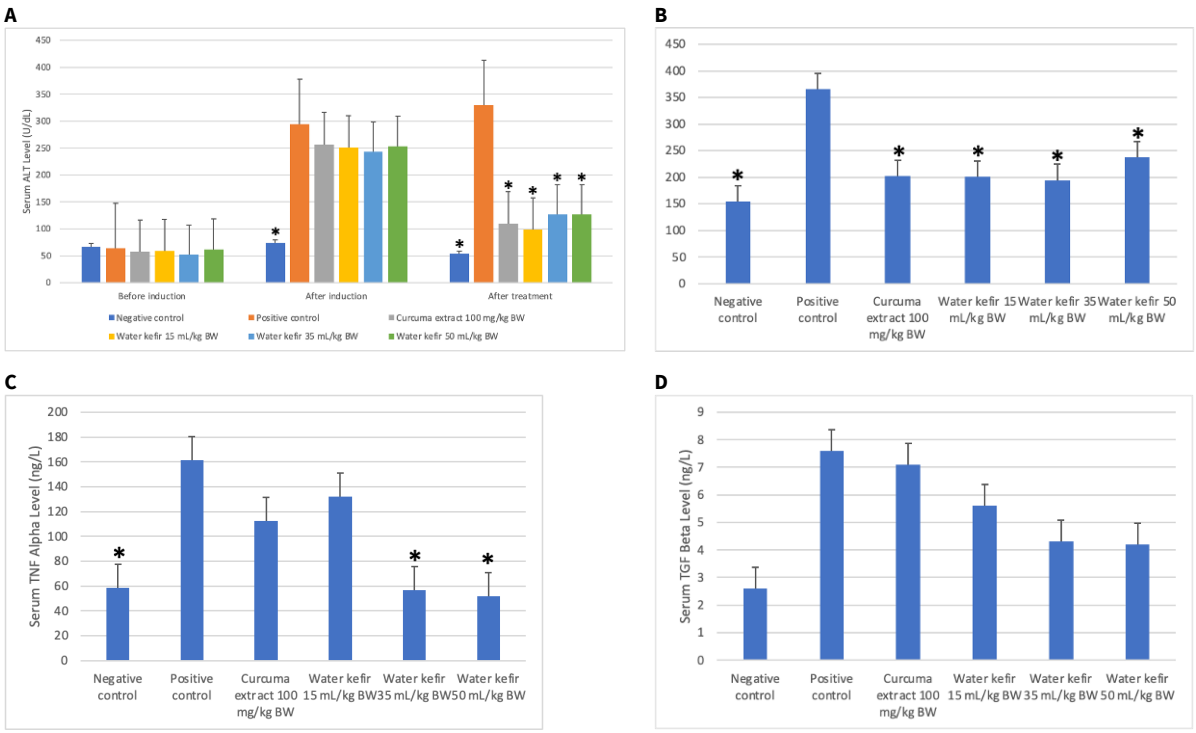

Fig. 1. (A) Before induction, after induction, and after treatment serum ALT level; **(B)** After treatment serum AST level; **(C)** After treatment serum TNF-α level; **(D)** After treatment serum TGF-β level.

Data represent mean ± SEM (n = 4). *p<0.05 significantly different result when compared to the positive control group.

Statistical analysis

All of the information is displayed in the form of individual data points as well as the mean along with the standard error of the mean (SEM). The statistical analysis was carried out with the help of Minitab software (version 19.0), and to make comparisons between several different groups, a one-way analysis of variance with Dunnett's post hoc test was utilized. All statistical graphs were created with Microsoft Excel 2019 in their respective versions. The level of significance that were considered to have been reached was $p<0.05$.

RESULTS

In vivo evaluation of hepatoprotective activity

The serum ALT levels, as the main parameter for the liver damage, were measured prior to and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment AST, TNF-α, and TGF-β levels were also evaluated. These findings were analyzed using a one-way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant rise in blood ALT levels demonstrated the establishment of an animal model with liver injury, according to statistical analysis, following the administration of CCl_4. When compared to the positive control group, ALT serum levels decreased significantly after two weeks of therapy with curcuma extract or water kefir ($p<0.05$). The three doses of water kefir groups demonstrated equivalent activity when curcuma extract was used as the standard treatment, and there was no significant difference between the three doses of water kefir ($p>0.05$). When compared to the positive control group, AST levels were also reduced dramatically following treatment with curcuma extract or water kefir. TNF-α levels in the water kefir group were significantly lower than in the positive control group at dosages of 35 and 50 mL/kg body weight ($p<0.05$). Even though there was no statistically significant difference in TGF-β levels, the group that received the treatment demonstrated a decrease in TGF-β levels.

A histological examination of a normal liver group revealed a typical central vein bordered with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct cell borders and sinusoidal gaps (Fig. 2A-B). The CCl_4-induced group developed centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig. 2C-D). The groups that received either *Curcuma* extract or water kefir treatment improved in varied necrotic conditions ranging

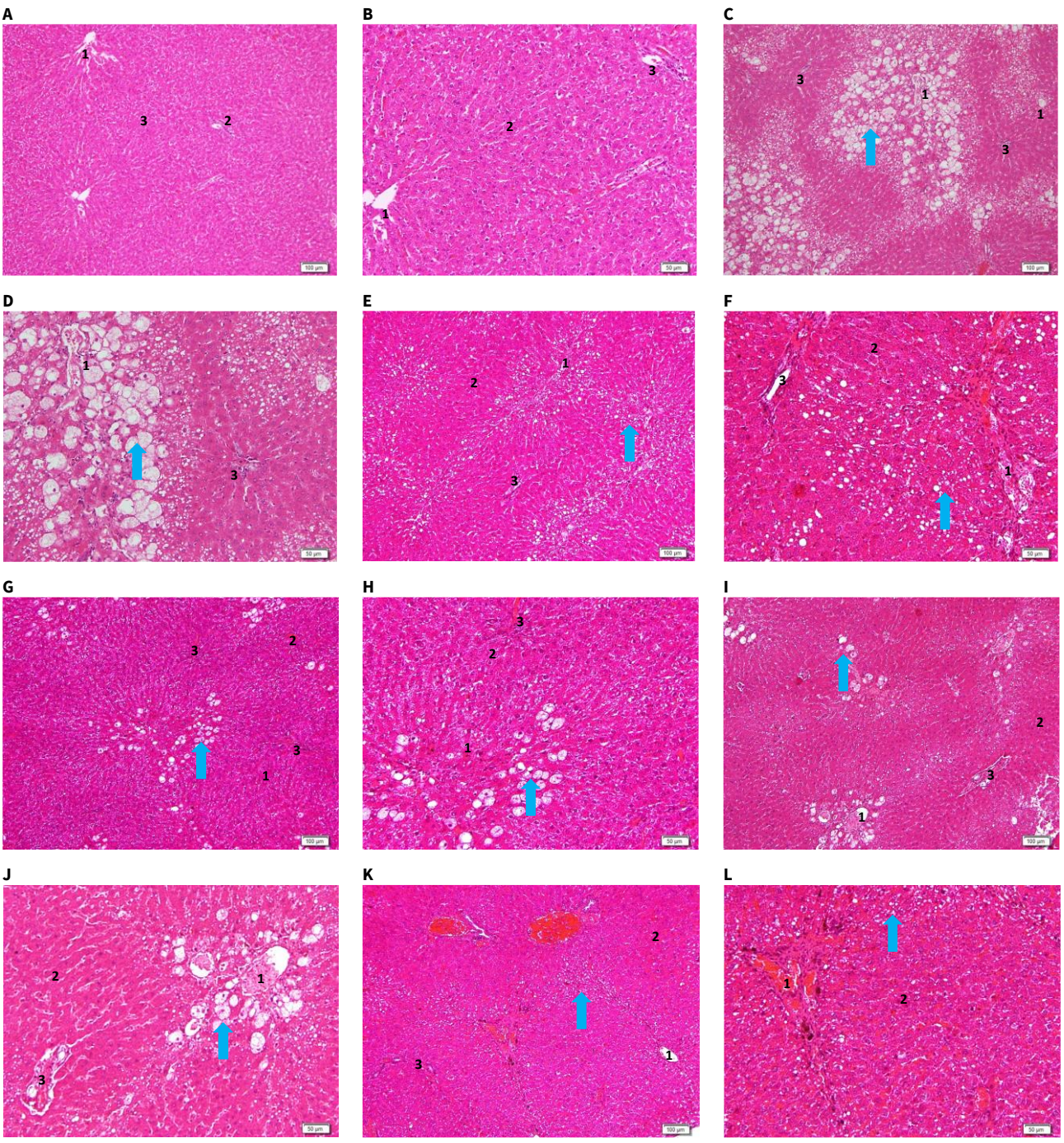

Fig. 2. Liver histology after CCl_4 intoxication and treatment with HE staining.

(A-B) Negative control group; **(C-D)** positive control group; **(E-F)** *Curcuma* extract group; **(G-H)** water kefir 15 mL/kg BW; **(I-J)** water kefir 30 mL/kg BW; and **(K-L)** water kefir 50 mL/kg BW. 1: central vein; 2: normal liver epithelial cell; 3: portal track; blue arrow: necrosis and liver epithelial cell vacuolization at centrolobular region.

from level 1 (water kefir 50 mL/kg BW) (Fig. 2K-L) to level 2 [*Curcuma* extract (Fig. 2E-F), water kefir 15 mL/kg BW (Fig. 2G-H), and water kefir 30 mL/kg BW (Fig. 2I-J)].

Molecular docking

Molecular docking studies are considered a powerful tool for predicting the potential targets of bioactive molecules. In order to carry out molecular docking simulations, one of the most critical steps is to identify the target active site. If the target protein is crystallized with a native ligand, in many instances, the location of the active site can be established without any difficulty (Li et al., 2019b). However, the NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) proteins do not have a native ligand, so the active site

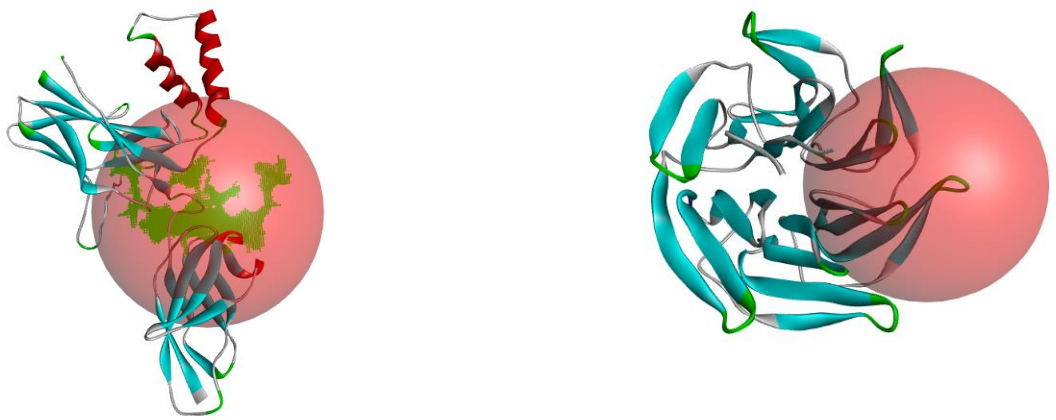

Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket.
(A) NF-κB (PDB ID 1A3Q) and **(B)** Keap1 (PDB ID 4L7B).

was determined. Active site prediction in docking is a computational method for predicting the location and orientation of a receptor protein's binding site for a ligand molecule. The active site prediction was based on a protein structural analysis and the identification of amino acid residues that are likely to interact with the ligand. The projected binding site is then utilized as a starting point for molecular docking, a computer method for predicting a ligand molecule's binding affinity and orientation to a receptor protein. The active site prediction for target proteins (Keap1 and NF-κB) gives the grid box coordinates (x y z) of 17.500880 Å, 62.323000 Å, and 0.973748 Å; and -1.751769 Å, -20.743853 Å, and -29.010438 Å, respectively (Fig. 3).

The docking results indicated that the 25 metabolites could interact with the target proteins (Keap1 and NF-κB) (Table 1). In general, all metabolites could interact with both NF-κB receptors (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). The interaction of metabolites with nuclear factor-kappa B receptors (PDB ID 1A3Q) revealed that 13 metabolites possessed free energy for binding that was greater than that of curcumin. On the other hand, there was not a single metabolite that had a free energy binding that was more powerful than that of nrf2 Keap1 (PDB ID 4L7B) (Table 1).

For volatile compounds, 2-phenyl ethanol and benzaldehyde interacted most strongly with the NF-κB receptor (PDB ID 1A3Q) with almost the same binding energy of -4.19 kcal/mol. As for organic acids, succinic acid, fumaric acid, and citric acid provided nearly the same strong interactions. Bond energy values of fumaric acid, succinic acid, and citric acid were -6.66, -6.24, and -6.25 kcal/mol, respectively. In sugars, glucose provided the strongest interaction with the NF-κB receptor (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2 Keap1 receptor (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to interact most strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46 kcal/mol. As for organic acids, succinic acid provided nearly the same strong interaction with a bond energy value of -3.02 kcal/mol, respectively. In sugars, glucose showed the strongest interaction with the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

The theoretical binding modes of the top three metabolites with their target proteins (Keap1 and NF-κB) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested that these metabolites interacted with the Keap1 and NF-κB to form a complex through hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site of NF-κB was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid residues of PRO223, LYS252, and LYS252, respectively. The interaction of benzaldehyde with the active site of NF-κB was formed by a hydrogen bond with the amino acid residue of LYS252. The interaction of fumaric acid with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction of glucose with the active site of NF-κB was formed by six hydrogen bonds with the amino acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were able to be formed by six hydrogen bonds, different types of amino acid residues were involved in the interaction, so fumaric acid interacted more strongly with the active site of NF-κB (Fig. 4).

Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-κB (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B).

No.	Metabolites	PDB: 1A3Q		PDB: 4L7B	
		Free energy of binding, ΔG (kcal/mol)	Inhibition constant, Ki (μM)	Free energy of binding, ΔG (kcal/mol)	Inhibition constant, Ki (μM)
	Volatile compounds				
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-4.19	851.52	-3.12	5,140
4	Ethyl acetate	-3.19	4,590	-2.43	16,670
5	2-Methylbutanal	-3.26	4,050	-2.35	19,050
6	1-Octanol	-2.65	11,490	-2.11	28,220
7	2-Phenylethyl acetate	-3.99	1,190	-3.33	3,590
8	Benzyl acetate	-4.16	887.66	-3.44	3,020
9	Benzaldehyde	-4.19	853.74	-3.19	4,550
10	Furfuryl acetate	-3.60	2,320	-3.32	3,660
11	Isobutyl acetate	-3.19	4,610	-2.94	7,020
12	Isopentyl acetate	-3.20	4,510	-3.05	5,820
13	2-Phenylacetaldehyde	-4.07	1,030	-3.46	2,930
14	3-Methylbutanal	-3.16	4,850	-2.73	10,020
15	Gluconic acid	-3.11	5,280	-1.63	64,290
	Organic acids				
16	Lactic acid	-4.92	249.28	-2.06	30,680
17	Succinic acid	-6.24	26.68	-3.02	6,140
18	Fumaric acid	-6.66	13.22	-2.45	16,100
19	Citric acid	-6.25	26.18	-1.87	42,440
20	Malic acid	-5.65	72.69	-2.53	13,860
21	Acetic acid	-4.99	219.33	-2.44	16,280
22	Ethanol	-2.39	17,800	-2.08	29,800
	Sugars				
23	Sucrose	-1.40	93,710	-1.33	105,320
24	Glucose	-3.71	1,900	-3.09	5,440
25	Fructose	-3.21	4,400	-2.65	11,440
	Reference compound				
	Curcumin	-3.44	2,990	-4.22	811.36

The interaction of 2-phenylacetaldehyde with the active site of Keap1 was formed by one hydrogen bond and one Pi-Alkyl interaction with amino acid residues of GLN530 and ALA556, respectively. The interaction of succinic acid with the active site of Keap1 was formed by four hydrogen bonds with the amino acid residues of ARG336, HIS575, and PHE577. The interaction of glucose with the active site of Keap1 was formed by six hydrogen bonds with the amino acid residues LYS551, ARG553, and ASP573. Although glucose can form more hydrogen bonds than 2-phenylacetaldehyde, the different types of amino acid residues involved have not been able to have a significant effect on the binding energy of its interaction with the active site of Keap1 (Fig. 5).

Curcumin (the reference compound) created three hydrogen bonds with the amino acid residues ARG52,

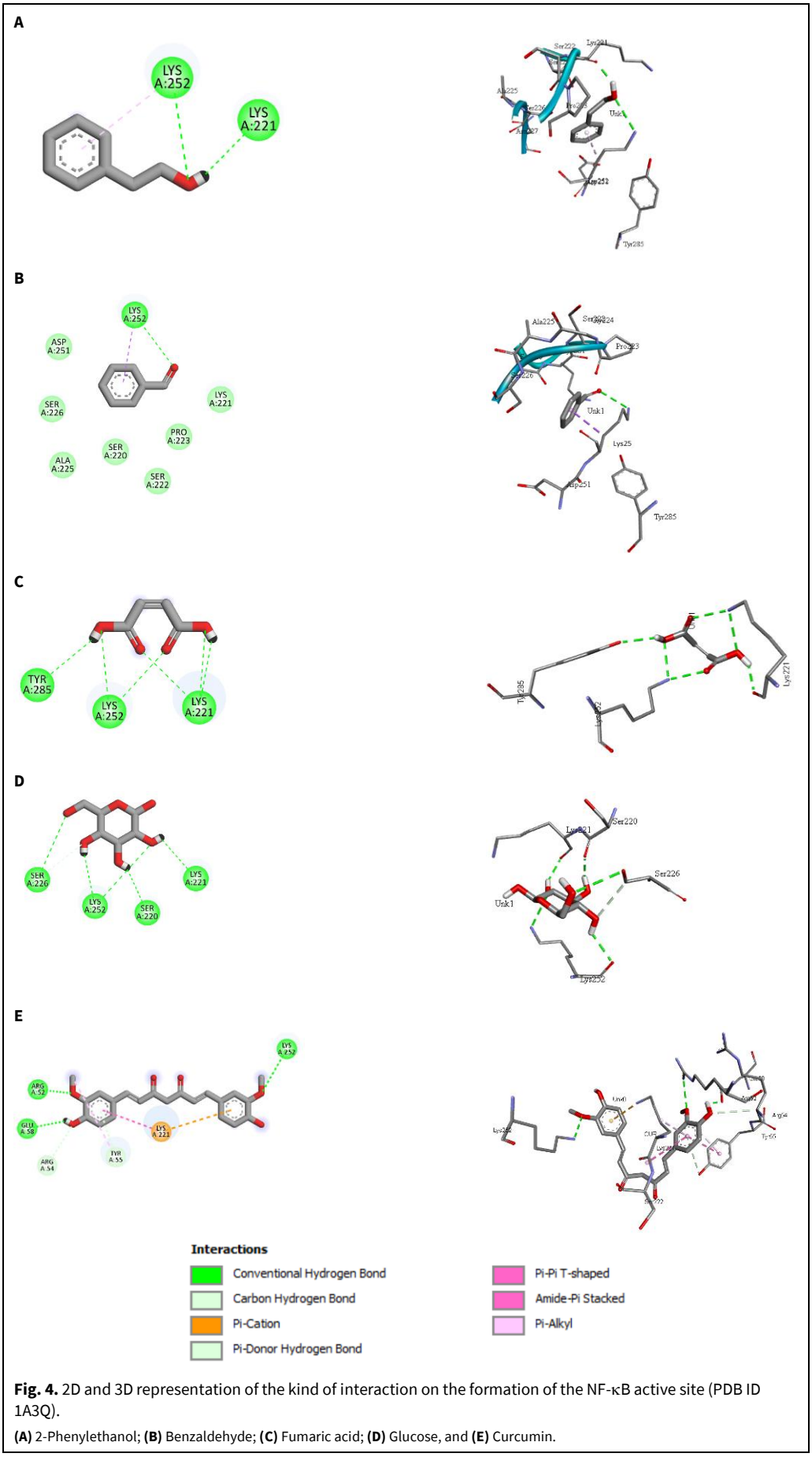

Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q).

(A) 2-Phenylethanol; **(B)** Benzaldehyde; **(C)** Fumaric acid; **(D)** Glucose, and **(E)** Curcumin.

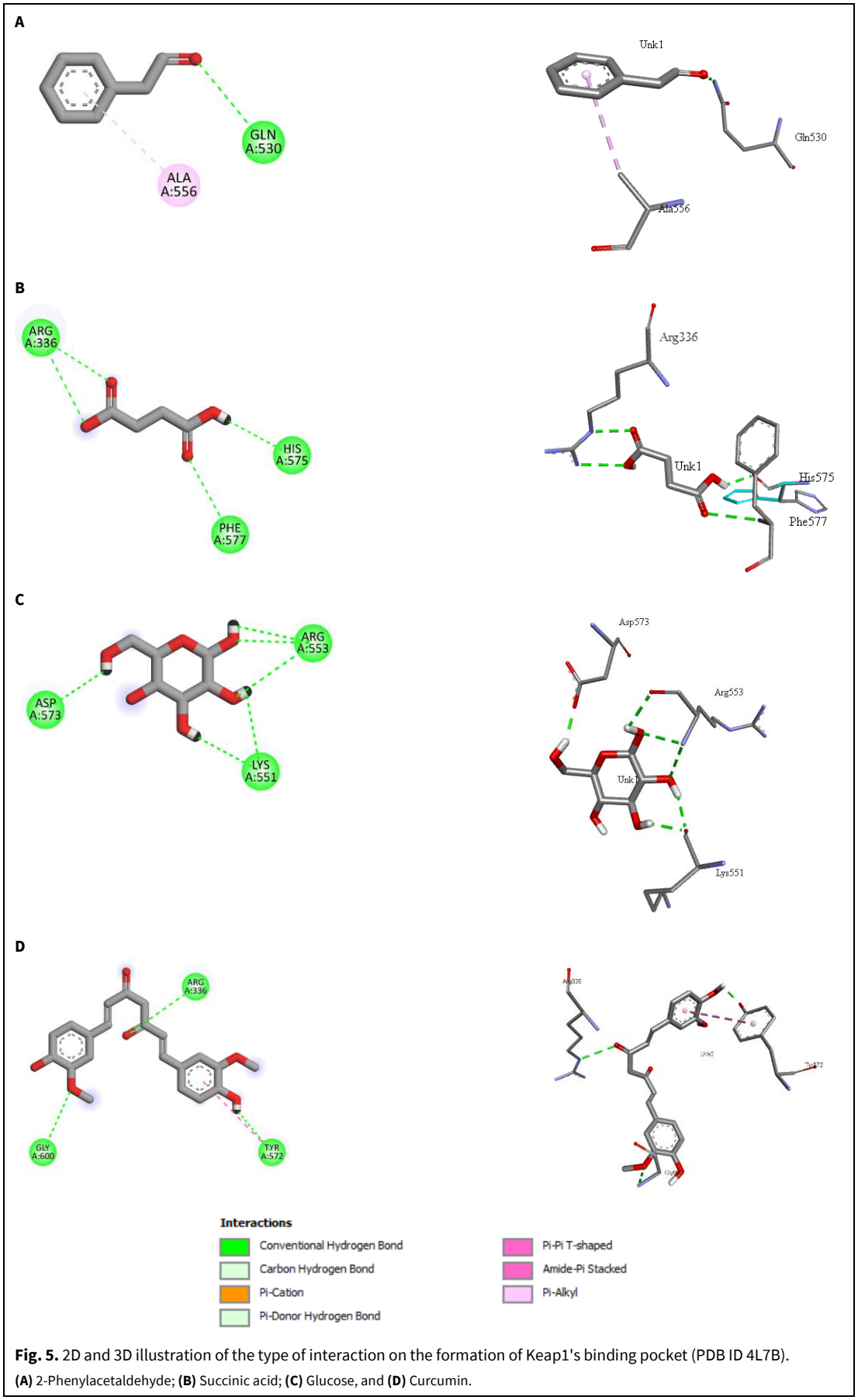

Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B).
(A) 2-Phenylacetaldehyde; **(B)** Succinic acid; **(C)** Glucose, and **(D)** Curcumin.

GLU58, and LYS252 to interact with the active site of NF-κB. Meanwhile, curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572, and GLY600 to interact with the active site of nrf2 Keap1 (Figs. 4 and 5). Despite the fact that curcumin could create three hydrogen bonds at both the active sites of NF-κB and nrf2 Keap1, its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol, benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to interact with NF-κB and nrf2 Keap1 receptors. Whereas fumaric acid and 2-phenylacetaldehyde were metabolites that had the strongest interaction with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

DISCUSSION

Increased liver enzyme production is one of the abnormalities indicating liver damage. This increase in secretion increases the leakage of enzymes into the circulation. Transaminase is a frequent enzyme used to evaluate liver disorders. The transaminases AST and ALT catalyze the transfer of the α-amino group from alanine and aspartic acid to α-ketoglutaric acid. AST is found in the liver, heart, and skeletal muscle, as well as the kidney, brain, pancreas, lung, leukocytes, and erythrocytes. While ALT is predominantly found in the liver, it is also found in low concentrations in other tissues (Lee et al., 2012). Consequently, ALT was used as the principal hepatotoxicity criterion in this study.

Carbon tetrachloride (CCl_4) is a component of the hepatotoxin, which acts after metabolic activation and is extensively employed as a liver-damaging agent. In this study, the administration of CCl_4 to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administrations. CCl_4 is metabolized by the enzyme cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl peroxyl radical, which rapidly reacts with lipids to form lipid peroxidation products. Free radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased protein synthesis, and increased oxidase activity. CCl_4 hepatotoxicity is characterized by hepatocellular necrosis with fat deposition (Ritesh et al., 2015). At the molecular level, administration of CCl_4 can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (Weber et al., 2003).

In terms of its pathophysiological underpinnings, liver illness is linked to a condition known as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Laureys and De Vuyst, 2017; Romero-Luna et al., 2020; Zavala et al., 2016). Both qualitative and quantitative changes in the gut microbiome have the potential to affect the composition of products produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et al., 2020). Qualitative changes include an imbalance between harmful and helpful microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of microbial products can all be caused by dysbiosis (Laureys et al., 2018). For this reason, the condition of the gut microbiome is an important factor in the initiation and development of chronic liver disease (Lee et al., 2021). Based on the results of the study, treatment with water kefir for two weeks after the occurrence of liver damage was able to improve the overall condition of the liver, which was marked by a significant decrease in the values of AST, ALT, TNF-α, TGF-β, and significant improvement in liver histology.

Water kefir contains a number of microorganisms that have been linked to health benefits, such as the probiotics *L. paracasei* and *B. cereus* (Fijan, 2014). This activity is linked to an increase in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4). Improvements in intestinal barrier function and histological integrity were also observed. Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs simultaneously with the restoration of the p38 MAPK pathway (Fijan, 2014; Yao et al., 2019; Tsai et al., 2020; Ji et al., 2022). Bacillus is a kind of endospore-forming bacterium that can endure extremely cold temperatures and lengthy periods of storage without dying; its spores can even tolerate the acidic environment of the stomach and make it all the way to the small intestine (Elshaghabee et al., 2017). *Bacillus cereus* has been shown to reduce ALT levels, an indicator of liver healing, in various animal models of liver injury. It protects the liver by reducing inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines (Kim et al., 2018; Li et al., 2019a; Xue et al., 2020). Also, when *Bacillus* spores were used first, hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al., 2020).

Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-negative acetic

acid bacteria have been found. One of the features that sets acetic acid bacteria apart from others is their alkaline-stable lipid membrane (Lynch et al., 2021). Their "oxidative" fermentation metabolism is responsible for the principal metabolic process in these bacteria, the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond, fermentation helps mediate the transition of diverse substrates into products. Although lactic acid bacteria have been studied more extensively than acetic acid bacteria (Hong et al., 2021; Semjonovs et al., 2014), various studies have shown promising results concerning the pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the severity of liver injury in rats with septic shock by increasing the expression of TRIM40. TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of cytokines such as IL-6 and TNF-α, raise the expression of IL-10, improve survival in septic mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased inflammation as well as the production of inflammatory cytokines (Yang et al., 2019). Acetic acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation and reduced hepatic lipid and body fat deposition (Kondo et al., 2009; Li et al., 2018).

Apart from microorganisms that directly provide hepatoprotective effects, the metabolites produced from these microorganisms also have the potential to be hepatoprotective. Molecular docking is a technique that is utilized in the context of NF-κB and Nrf2 to make predictions regarding the binding affinity and orientation of small-molecule inhibitors to their active sites. The transcription factor known as NF-κB is an essential component in the management of both the immune system and the inflammatory response (Dai et al., 2021). The expression of important inflammatory genes can be inhibited by small-molecule inhibitors that impair the interaction between NF-κB and DNA. These inhibitors have the potential to be used in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (Jiang et al., 2019; Zhao et al., 2017). Small-molecule inhibitors that disrupt the link between Keap1 and Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be disrupted by small-molecule inhibitors (Zhao et al., 2017).

Binding energy and Ki are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor protein. Binding energy is the energy released when a ligand binds to a receptor protein, and it is calculated as the difference between the energy of the bound complex and the energy of the unbound ligand and protein (Meng et al., 2011). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites. Both binding energy and Ki are used to predict the binding affinity and selectivity of a ligand to a receptor protein (Du et al., 2016). Ki, on the other hand, is the dissociation constant of the ligand-receptor complex. It shows how much ligand is needed to bind 50% of the receptor binding sites (Du et al., 2016).

Based on the results of an *in silico* study of water kefir metabolite compounds, it was known that fumaric acid and 2-phenylacetaldehyde have the strongest interactions with NF-κB (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively. Fumaric acid has been studied for its potential as a hepatoprotective agent. Fumaric acid protects rat livers against cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic injury against cadmium in rats (Kaur et al., 2020). Fumaric acid esters were found to ameliorate inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic fat accumulation (Šilhavý et al., 2014).

NF-κB and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF-κB is involved in the inflammatory response, and Nrf2 is involved in the antioxidant response (Ganesh Yerra et al., 2013; Wang et al., 2022a). Both transcription factors have been investigated as potential targets for the development of hepatoprotective agents (Gao et al., 2022; Li et al., 2023; Rahman et al., 2021; Wang et al., 2022a). There is evidence of crosstalk between the Nrf2 and NF-kB pathways (Ganesh Yerra et al., 2013; Gao et al., 2022). The Nrf2 pathway inhibits the activation of the NF-κB pathway by increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and detoxifies (Ganesh Yerra et al., 2013). The crosstalk between Nrf2 and NF-κB could be a new therapeutic target against hepatotoxicity (Gao et al., 2022). Researchers have tried to identify molecule activators of Nrf2 as chemoprevention for ROS-dependent carcinogenesis, while others have focused on identifying Nrf2 inhibitors to increase the sensitivity of cancer cells to chemotherapy (Sharifi-Rad et al., 2023). While NF-κB and Nrf2 are involved in different cellular processes, they have both been investigated as potential targets for the development of hepatoprotective

agents. Molecular docking studies have been used to investigate the interaction of potential hepatoprotective agents with these transcription factors. There is also evidence of crosstalk between the Nrf2 and NF-κB pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF-κB plays a crucial role in regulating inflammation and cell death (Luedde and Schwabe, 2011). In response to many stimuli that may pose a threat to the host, NF-κB is activated, setting in motion processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde and Schwabe, 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs) include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory cytokines, including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe, 2011). When NF-B is activated, a lot of genes with B-binding sites are transcribed. These genes play important roles in controlling inflammation, the immune response, and cell survival. In an NF-κB-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by reducing BAMBI expression (an inhibitory TGF-β pseudoreceptor) and increasing Kupffer cell chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF-β, which stimulates HSCs unrestrictively. When HSCs have been activated, NF-κB serves a second crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine way, all play roles in activating NF-κB in activated hepatic stellate cells. More activated HSCs and extracellular matrix are deposited in the liver as a result of greater HSC activation and survival (Luedde and Schwabe, 2011).

TNF-α and TGF-β can stimulate one another's production, and TNF-α has been shown to influence TGF-β expression in a variety of cells and tissues (Liu et al., 2022). TNF-α is an inflammatory cytokine that contributes to liver inflammation, and chronic liver inflammation results in liver fibrosis. TNF-α exerts its effects on liver fibrosis via multiple mechanisms, including TGF-β signaling activation (Yang and Seki, 2015). Targeting TNF-α and TGF-β signaling pathways may, therefore, have therapeutic potential for treating liver diseases. In regard to hepatoprotective effects, the relationship between TGF-β and TNF-α is complex and not completely understood.

CONCLUSION

This study evaluated the hepatoprotective qualities of Indonesian water kefir in rats with CCl_4-induced liver damage. Water kefir administration improved the condition of liver damage, characterized by a decrease in serum levels of AST, ALT, TNF, TGF, and an improvement of liver tissue profile. *In silico* evaluation showed that the metabolites in water kefir were able to interact with target proteins in the NF-κB and Nrf2 pathways. It was concluded that water kefir improves the condition of the liver by reducing the level of necrosis and fibrosis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Aligita W	Singgih M	Sutrisno E	Adnyana IK
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x			x
Experimental studies	x			x
Data acquisition	x			x
Data analysis	x			x
Statistical analysis	x			x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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