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6 Hepatoprotective Study of Indonesian Water Kefir Against 7 CCl₄-Induced Liver Injury in Rats

8
9 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
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

29

30 ABSTRACT

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

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

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

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

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

44
45 Keywords: Hepatoprotective; Molecular docking; Metabolites; NF-κB; Nrf2; Water kefir

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48 INTRODUCTION

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

61 Water kefir has been used medicinally for a very long time, and recent research has
62 indicated that it may have a variety of positive effects on people's health. It has been
63 demonstrated that water kefir contains non-pathogenic bacteria, in conjunction with the
64 production of organic acids, can inhibit the growth of pathogenic microbes, such as *Shigella*
65 *sp.*, *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; and also, filamentous fungi such as
66 *Aspergillus ochraceus*, *A. niger*, *A. flavus*, *Penicillium sp.*, and *Rhizopus sp.* (add bibliography(ies))
67 In addition to its antibacterial properties, water kefir possesses a broad spectrum of
68 pharmacological effects. Some of these therapeutic effects are anti-inflammatory (5,6),
69 antioxidant (6-8), hepatoprotective (9,10), antihyperglycemic and antihyperlipidemic (11,12),
70 anti-edematous (13), antitumor (14), antihypertensive (15), immunomodulant (16), and anti-
71 ulcerogenic (17). However, no studies have been reported on the hepatoprotective effects of
72 water kefir against carbon tetrachloride (CCl₄)-induced liver injury.

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

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

86 Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while
87 lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage.
88 Additionally, a variety of aromatic and volatile compounds are produced, including ethyl
89 acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others
90 (compared to their threshold levels) (21). The chemical constituents of both phytochemicals
91 and secondary metabolites in natural products, including water kefir, are certainly capable of
92 providing various pharmacological effects for the body (22,23). However, an *in silico* study to
93 evaluate the metabolite content in water kefir has not been reported yet. Because of its capacity
94 to speed up the process of identifying and optimizing lead compounds, the *in silico* method
95 has become the front-runner in the race to improve the speed and accuracy of the process of
96 discovering new drugs. This is because the *in silico* method can identify and optimize lead
97 compounds more quickly. Techniques such as molecular docking and molecular dynamics
98 (MD) were able to directly indicate a small number of compounds that have high affinity and
99 selectivity by analyzing how the ligand and target interact with one another (24). Please, the
100 style for indicating bibliographic citations does not comply with JPPRes requirements, please
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102 There are numerous studies demonstrating the hepatoprotective activity of kefir. Therefore,
103 the authors should state here the novel effect they intend to find, which has not been reported
104 in the scientific literature. Please better state the research problem and how you intend to solve
105 it.

106 Therefore, the purpose of this study was to evaluate the hepatoprotective effects of water
107 kefir in CCl₄-induced rats while also investigating the stability interactions of its metabolites
108 within the NF- κ B and Nrf2 receptors using molecular docking study. Our findings suggest
109 that water kefir and its the metabolites it produces could be a promising therapeutic agent for
110 the management of liver injury that is both safe and effective. Please move this last sentence
111 to the Conclusions, it is not valid for an Introduction.

112

113 MATERIAL AND METHODS

114 Materials and Reagents

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

119 Experimental sample and reference extract

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

127 **Animals and Experimental Design**

128 Rats (Wistar strain, male, 200-250 g) were maintained on normal pellet food and tap water (*ad*
129 *libitum*). Four mice in each group were used. All procedures relating to animals and their care
130 conformed to the international guidelines Principles of Laboratory Animals Care (NIH
131 publication no. 85-23, revised 1985). In order to develop an animal model with liver injury, the
132 rats received CCl₄ (20% in olive oil) 1.25 mL/kg BW every 2 days via a gavage tube (25). The
133 rats were randomized into five groups after the development of animals with liver injury,
134 which is characterized by a significant increase in serum ALT level, as follows: (1) positive
135 control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group,
136 (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group; with addition
137 (6) negative control group. Each group received group-specific treatment for two weeks, along
138 with administration of 1.25 mL/kg BW of CCl₄ (20% in olive oil) every three days. Serum ALT
139 level, as the main parameter, was measured prior to induction, following induction, and
140 following treatment. Meanwhile, following therapy, serum AST, TNF- α , TGF- β levels, and
141 liver histopathological were evaluated. Please indicate how the serum was extracted in the
142 rats? from which part of the animal and how the blood was extracted. How were AST, TNF-
143 α , TGF- α levels measured? give details of this. How was the liver extracted from the animals?
144 how was the liver histopathological analysis performed.

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

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

154

155 **Molecular docking simulation**

156 The PyRx software (26) was used to perform molecular docking experiments to anticipate
157 the manner of binding that happens between metabolites as small-molecule ligands and
158 biological macromolecules. The NCBI PubChem database (<https://pubchem.nlm.nih.gov/>,
159 accessed on 03 May 2023) was used to derive the three-dimensional structure of water kefir
160 metabolites (27). Target proteins like NF- κ B (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B)
161 were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/>, accessed on 03 May
162 2023). PyRx program was used to reduce protein, ligand converted to PDBQT (28), then
163 maximized GRID parameter (29) performed docking study (30). The BIOVIA Discovery Studio
164 2017 R2 program was used to view the protein and ligand complex and distance (31). The
165 BIOVIA Discovery Studio 2017 R2 tool was also utilized to find protein active sites.

166 **Statistical Analysis**

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

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

173 RESULTS

174 In vivo evaluation of hepatoprotective activity

175 The serum ALT levels, as the main parameter for the liver damage, were measured prior to
 176 and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment
 177 AST, TNF- α , and TGF- β levels were also evaluated. These findings were analyzed using a one-
 178 way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant
 179 rise in blood ALT levels demonstrated the establishment of an animal model with liver injury,
 180 according to statistical analysis, following the administration of CCl₄. When compared to the
 181 positive control group, ALT serum levels reduced significantly after two weeks of therapy
 182 with curcuma extract or water kefir. The three doses of water kefir groups demonstrated
 183 equivalent activity when curcuma extract was used as the standard treatment and there was
 184 no significant difference between the three doses of water kefir. When compared to the
 185 positive control group, AST levels were also reduced dramatically following treatment with
 186 curcuma extract or water kefir. TNF- α levels in the water kefir group were significantly lower
 187 than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though
 188 there was no statistically significant difference in TGF- β levels, the group that received the
 189 treatment demonstrated a decrease in TGF- β levels.

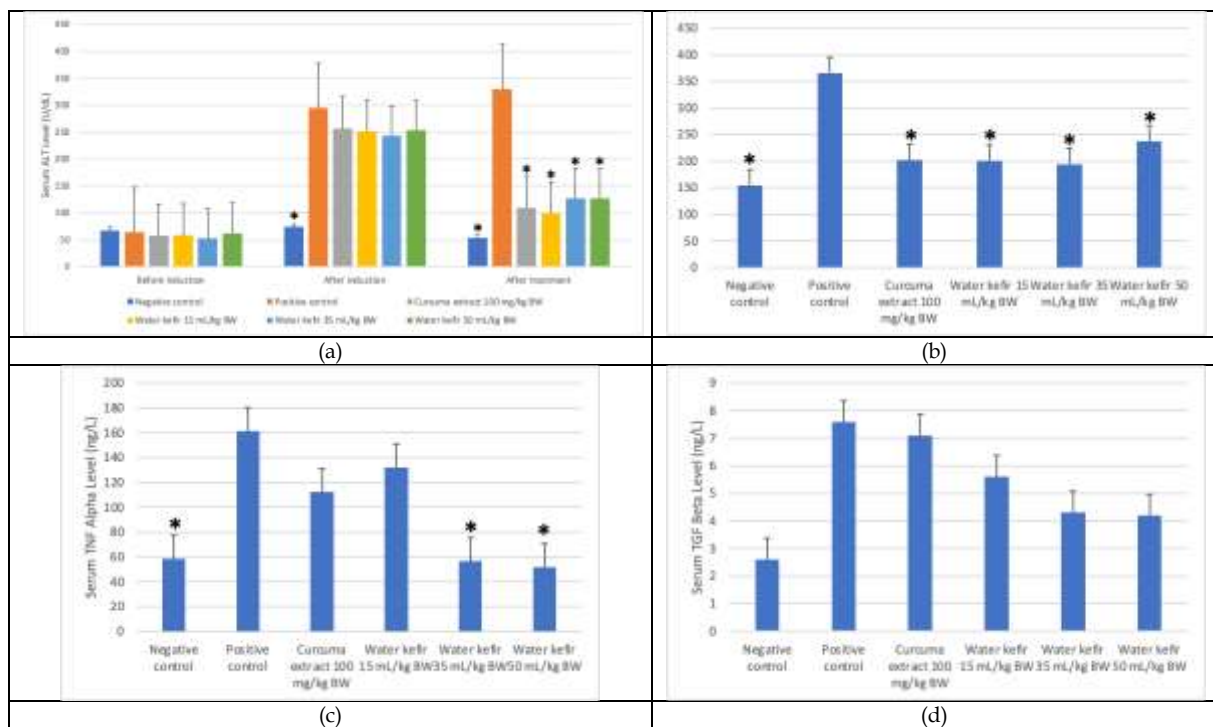


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF- α level; (d) After treatment serum TGF- β level. * indicates a significantly different result when compared to the positive control group; # indicates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

190

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

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

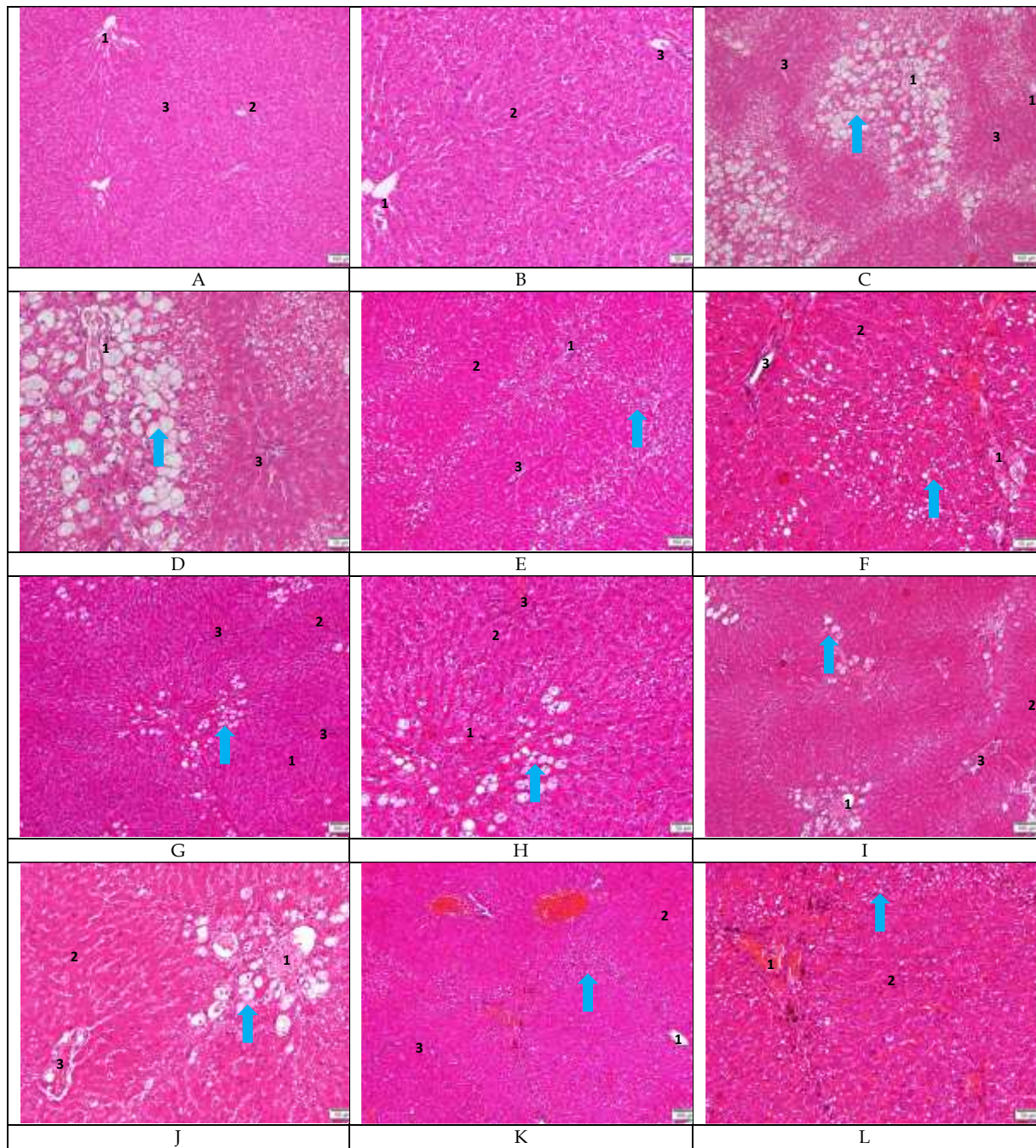


Fig. 2. Liver histology after CCl₄ intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal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

200

201 Molecular docking

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

205 native ligand, in many instances, the location of the active site can be established without any
 206 difficulty (32). However, the NF- κ B (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) proteins
 207 do not have a native ligand, so the active site was determined. Active site prediction in docking
 208 is a computational method for predicting the location and orientation of a receptor protein's
 209 binding site for a ligand molecule. The active site prediction is based on a protein structural
 210 analysis and the identification of amino acid residues that are likely to interact with the ligand.
 211 The projected binding site is then utilized as a starting point for molecular docking, a computer
 212 method for predicting a ligand molecule's binding affinity and orientation to a receptor
 213 protein. The active site prediction for target proteins (Keap1 and NF- κ B) gives the grid box
 214 coordinates (x y z) of 17.500880, 62.323000, and 0.973748; and -1.751769, -20.743853, and -
 215 29.010438, respectively (Fig. 3).

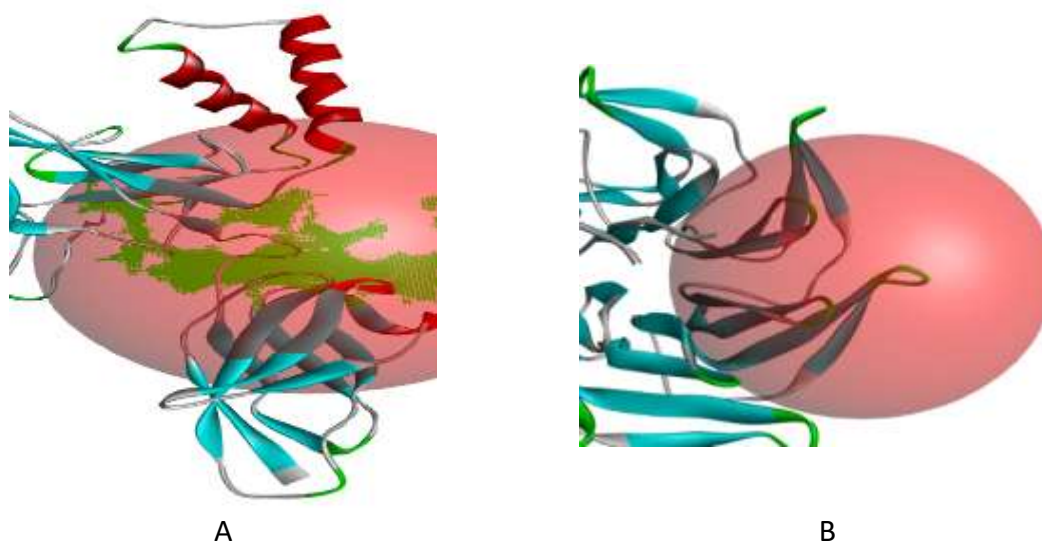


Fig. 3. Binding pocket (red color) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF- κ B (PDB ID 1A3Q) B) Keap1 (PDB ID 4L7B).

216
 217 The docking results of the 25 metabolites could interact with target proteins (Keap1 and
 218 NF- κ B) (Table 1). In general, all metabolites were able to form interactions with both NF- κ B
 219 receptors (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) (Table 1). For volatile compounds, 2-
 220 phenyl ethanol and benzaldehyde interact most strongly with the NF- κ B receptor (PDB ID
 221 1A3Q) with almost the same binding energy of -4.19 kcal/mol. As for organic acids, succinic
 222 acid, fumaric acid, and citric acid provide nearly the same strong interactions. Bond energy
 223 values of succinic acid, fumaric acid, and citric acid of -6.24, -6.66, and -6.25 kcal/mol,
 224 respectively. In sugars, glucose provides the strongest interaction with the NF- κ B receptor
 225 (PDB ID 1A3Q) where the binding energy was -3.71 kcal/mol. As for the nrf2 Keap1 receptor
 226 (PDB ID 4L7B), 2-phenylacetaldehyde from volatile compounds was able to interact most
 227 strongly with the Keap1 receptor (PDB ID 4L7B) with a binding energy of -3.46 kcal/mol. As
 228 for organic acids, succinic acid provides nearly the same strong interaction with a bond energy
 229 value of -3.02 kcal/mol, respectively. In sugars, glucose provides the strongest interaction with
 230 the Keap1 receptor (PDB ID 4L7B) where the binding energy was -3.09 kcal/mol.

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

No.	Metabolites	PDB: 1A3Q	PDB: 4L7B
		Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, K_i (μM)
			Free Energy of Binding, ΔG (kcal/mol)
			Inhibition Constant, K_i (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- κ B inhibitor/activator.					

233

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

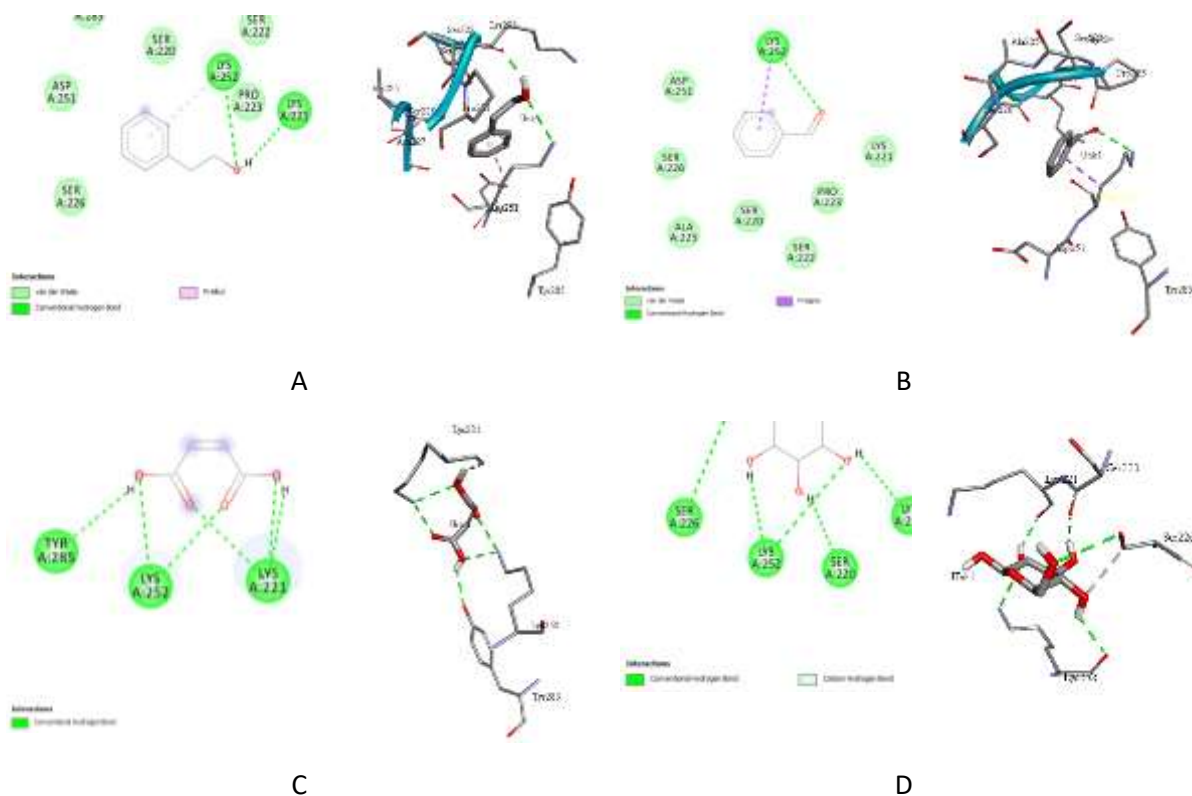
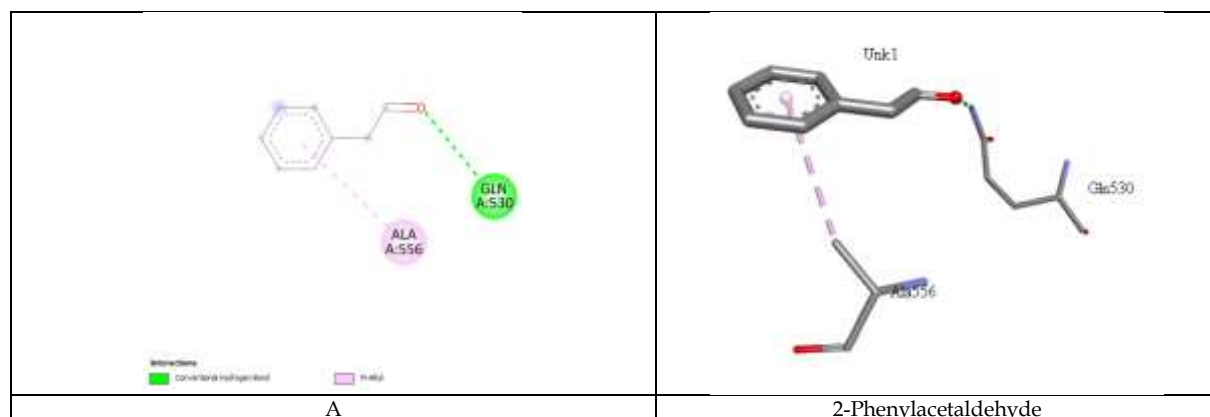


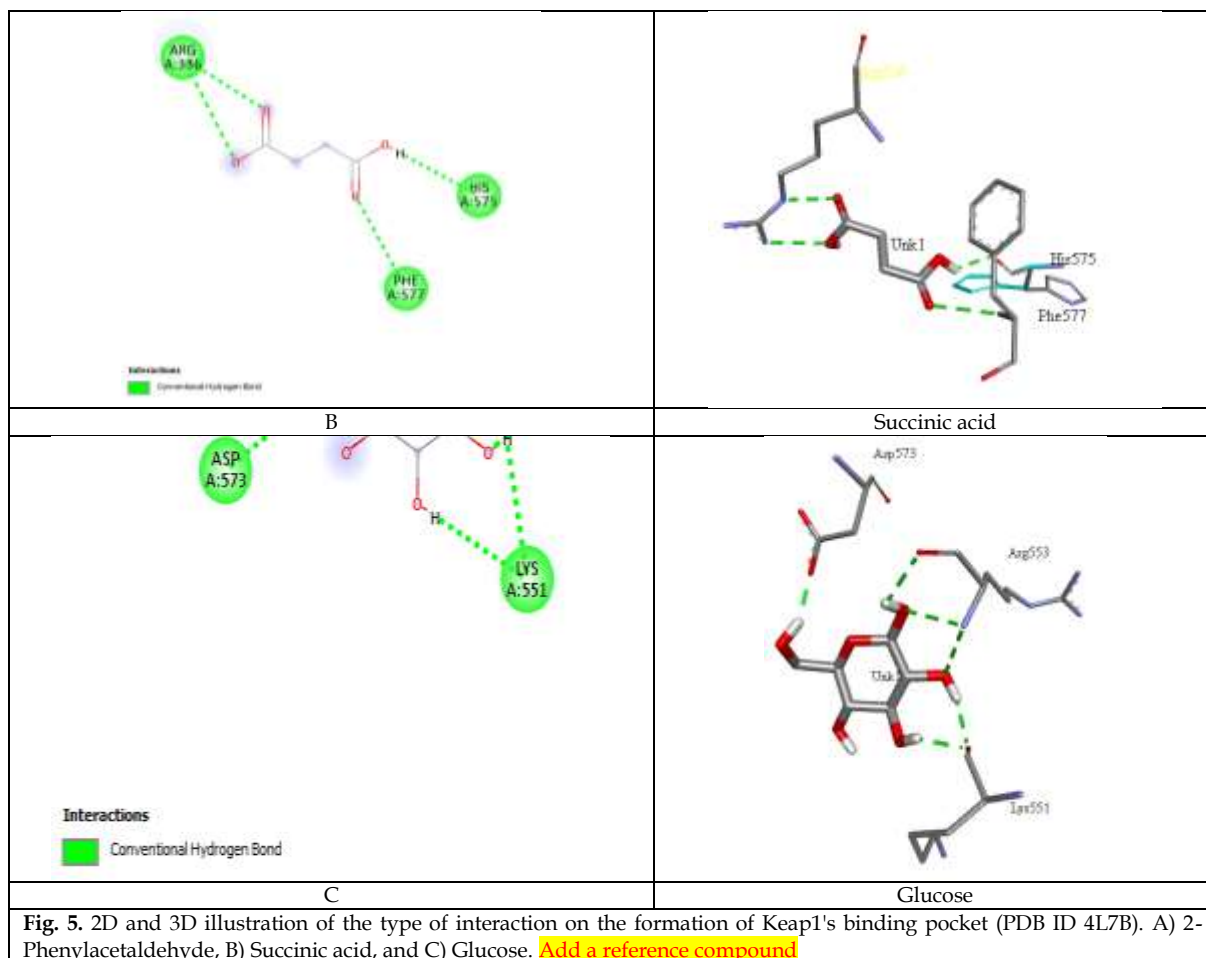
Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF- κ B active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, and D) Glucose. [Add a reference compound](#)

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).

258





259

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

264 DISCUSSION

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

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

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

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

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

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

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

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

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

356 Based on the results of an in silico study of water kefir metabolite compounds, it was known
357 that fumaric acid and 2-phenylacetaldehyde have strongest interaction with NF- κ B (PDB ID
358 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively (bibliography). Fumaric acid has
359 been studied for its potential as a hepatoprotection. Fumaric acid protected rat livers against
360 cadmium toxicity by decreasing oxidative stress and improving hepatic serum. Fumaric acid
361 prevented cadmium-accrued hepatocellular damage and showed the potential to avert hepatic
362 injury against cadmium in rats (60). Fumaric acid esters were found to ameliorate
363 inflammation and oxidative stress in rats, which was associated with less adiposity and ectopic
364 fat accumulation (61).

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

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

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

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

406 CONCLUSION

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

413 CONFLICT OF INTEREST

414 The authors declare no conflict of interest.

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612 **Please, insert here the Supplementary Data, if any.**
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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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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

23

24 ABSTRACT

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

28 Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl₄-induced acute liver injury and to in silico
29 investigate metabolites that play an important role in hepatoprotective mechanisms.

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

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

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

42
43 Keywords: Free radicals; Liver diseases; Kefir; Molecular docking simulation; Probiotics

44 45 INTRODUCTION

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

58 Water kefir has been used medicinally for a very long time, and recent research has
59 indicated that it may have a variety of positive effects on people's health. It has been
60 demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the
61 production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*,
62 *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus*
63 *ochraceus*, *A. niger*, *A. flavus*, *Penicillium sp.*, and *Rhizopus sp.* (Al-Mohammadi et al. 2021). In
64 addition to its antibacterial properties, water kefir possesses a broad spectrum of
65 pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al.
66 2003; Aligita et al. 2020), antioxidant (Alsayadi et al. 2013; Aligita et al. 2020; Darvishzadeh et
67 al. 2021), hepatoprotective (Aspiras et al. 2015; Aligita et al. 2021), antihyperglycemic and
68 antihyperlipidemic (Alsayadi et al. 2014; Rocha-Gomes et al. 2018), anti-edematous (Moreira
69 et al. 2008), antitumor (Zamberi et al. 2016), antihypertensive (Gamba et al. 2019),
70 immunomodulant (Calatayud et al. 2021), and anti-ulcerogenic (Rodrigues et al. 2016).
71 However, no studies have been reported on the hepatoprotective effects of water kefir against
72 carbon tetrachloride (CCl₄)-induced liver injury.

73 Studies have shown that acute liver injury is frequently accompanied by high levels of
74 oxidative stress and inflammatory responses (Dai et al. 2021a). These findings have been found
75 in several studies. The most important signaling pathways that are involved in the regulation
76 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- κ B 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- κ B 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 CCl₄-induced rats while also investigating the stability interactions of its metabolites within the NF- κ B and Nrf2 receptors using molecular docking studies.

108

109 MATERIAL AND METHODS

110 Materials and Reagents

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

115 Experimental sample and reference extract

116 The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution
117 was produced using a fermentation procedure. The initial stage involved the preparation of
118 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The
119 sugar and warm distilled water were mixed in a beaker, followed by the addition of water
120 kefir grains and raisins to the resulting sugar solution. The fermentation procedure was
121 conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

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

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

128 **Animals and Experimental Design**

129 Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water
130 *ad libitum*. Four mice in each group were used. All procedures relating to animals and their
131 care conformed to the international guidelines Principles of Laboratory Animal Care (NIH
132 publication no. 85-23, revised 1985) with the ethical approval number
133 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats
134 received CCl₄ (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al.
135 2011). The rats were randomized into five groups after the development of animals with liver
136 injury, which is characterized by a significant increase in serum ALT level, as follows: (1)
137 positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg
138 BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with
139 the addition (6) negative control group. Each group received group-specific treatment for two
140 weeks, along with the administration of 1.25 mL/kg BW of CCl₄ (20% in olive oil) every three
141 days.

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

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

155 **Molecular docking simulation**

156 Molecular docking experiments were done with the PyRx software (Dallakyan and Olson
157 2015) to predict how metabolites, which are small-molecule ligands, bind to biological
158 macromolecules. The NCBI PubChem database (<https://pubchem.nlm.nih.gov/>, accessed on
159 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites
160 (Patel et al. 2022). Target proteins like NF- κ B (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B)
161 were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/>, accessed on 03 May
162 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et
163 al. 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study
164 (Febrina et al. 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the
165 protein and ligand complex and distance (Ischak et al. 2023). The BIOVIA Discovery Studio
166 2017 R2 tool was also utilized to find protein active sites.

167 **Statistical Analysis**

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

174 **RESULTS**

175 ***In vivo* evaluation of hepatoprotective activity**

176 The serum ALT levels, as the main parameter for the liver damage, were measured prior to
 177 and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment
 178 AST, TNF- α , and TGF- β levels were also evaluated. These findings were analyzed using a one-
 179 way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant
 180 rise in blood ALT levels demonstrated the establishment of an animal model with liver injury,
 181 according to statistical analysis, following the administration of CCl₄. When compared to the
 182 positive control group, ALT serum levels decreased significantly after two weeks of therapy
 183 with curcuma extract or water kefir. The three doses of water kefir groups demonstrated
 184 equivalent activity when curcuma extract was used as the standard treatment, and there was
 185 no significant difference between the three doses of water kefir. When compared to the
 186 positive control group, AST levels were also reduced dramatically following treatment with
 187 curcuma extract or water kefir. TNF- α levels in the water kefir group were significantly lower
 188 than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though
 189 there was no statistically significant difference in TGF- β levels, the group that received the
 190 treatment demonstrated a decrease in TGF- β levels.

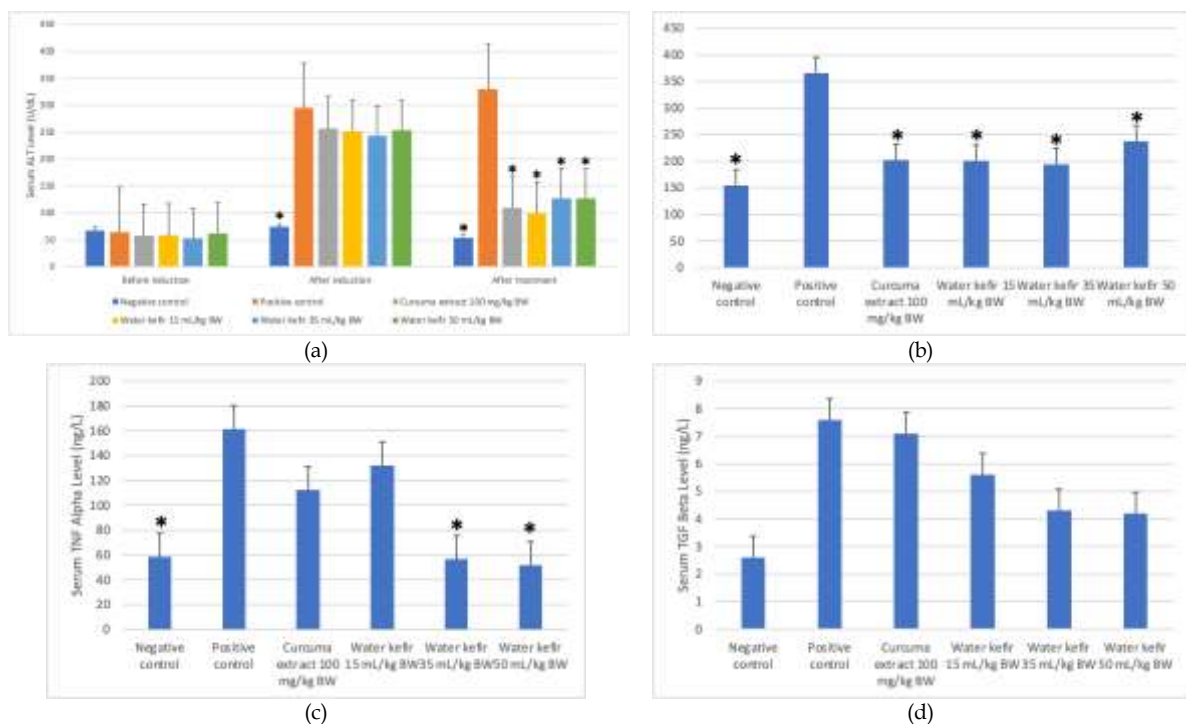


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF- α level; (d) After treatment serum TGF- β level. * indicates a significantly different result when compared to the positive control group; # indicates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

192 A histological examination of a normal liver group revealed a typical central vein bordered
 193 with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct
 194 cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl₄-induced group developed
 195 centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and
 196 necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The
 197 group that received either curcuma extract or water kefir treatment improved in varied
 198 necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2
 199 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir
 200 30 mL/kg BW (Fig 2I & 2J)).

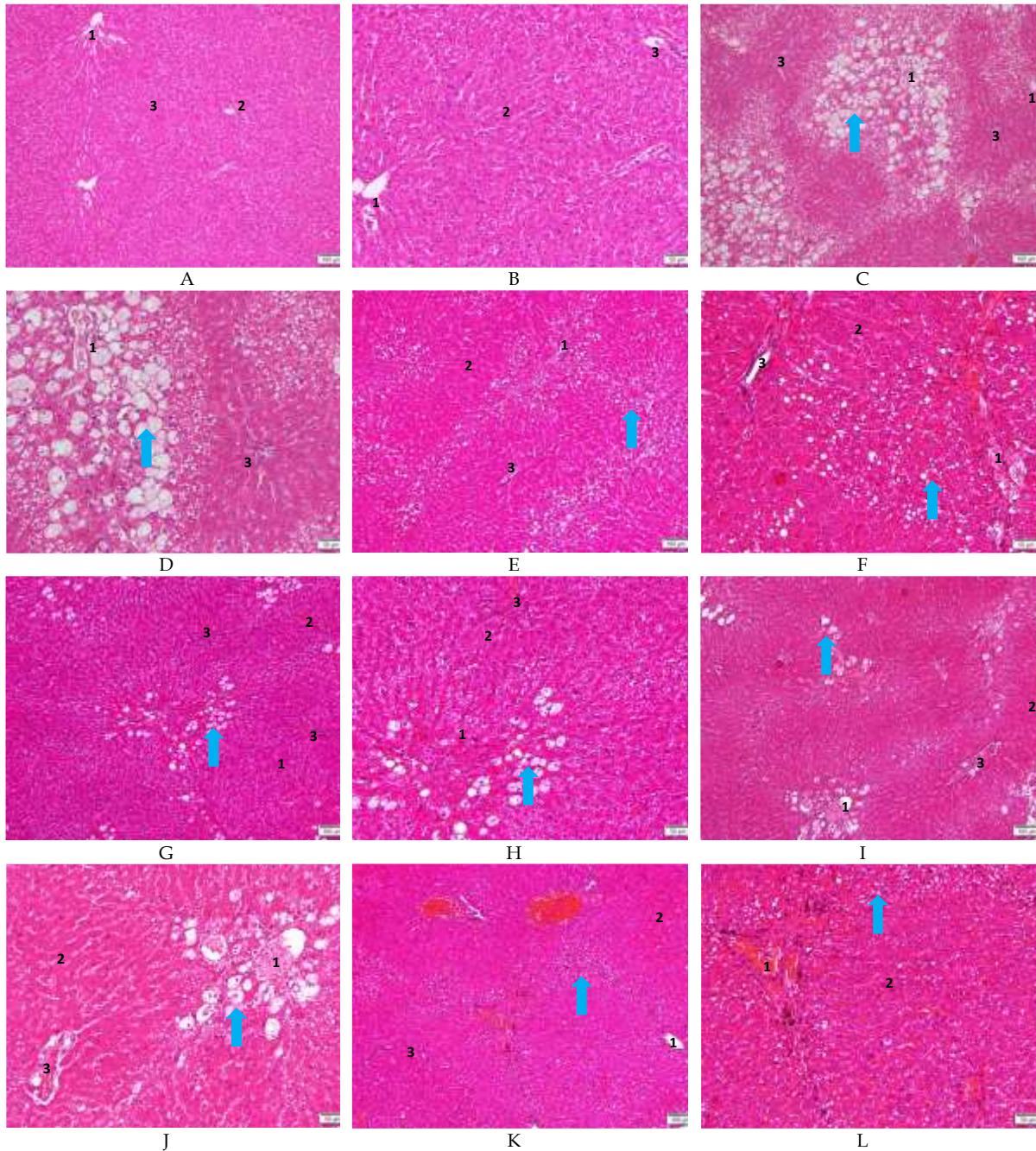


Fig. 2. Liver histology after CCl₄ intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal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

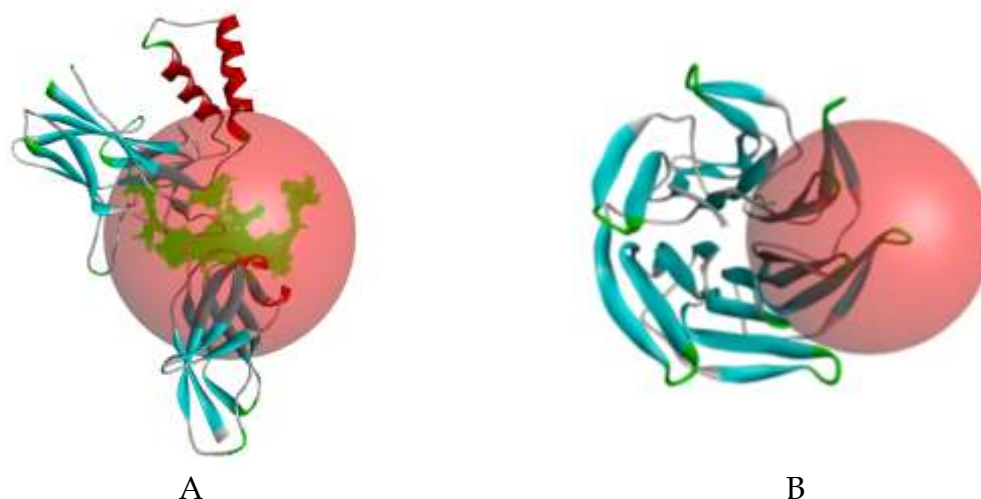


Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF- κ B (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

217

218 The docking results of the 25 metabolites could interact with target proteins (Keap1 and
219 NF- κ B) (Table 1). In general, all metabolites could interact with both NF- κ B 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- κ B 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- κ B 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

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

237

238

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

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

241

242 The theoretical binding modes of the top three metabolites with their target proteins (Keap1
 243 and NF- κ B) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested
 244 that these metabolites interacted with the Keap1 and NF- κ B to form a complex through
 245 hydrogen bonds with various residues. The interaction of 2-phenyl ethanol with the active site
 246 of NF- κ B was formed by two hydrogen bonds and one Pi-Alkyl interaction with amino acid
 247 residues of PRO223, LYS252, and LYS252, respectively. The interaction of benzaldehyde with
 248 the active site of NF- κ B was formed by a hydrogen bond with the amino acid residue of
 249 LYS252. The interaction of fumaric acid with the active site of NF- κ B was formed by six
 250 hydrogen bonds with the amino acid residues of LYS221, LYS252, and TYR285. The interaction
 251 of glucose with the active site of NF- κ B was formed by six hydrogen bonds with the amino
 252 acid residues SER220, LYS221, SER226, and LYS252. Although glucose and fumaric acid were
 253 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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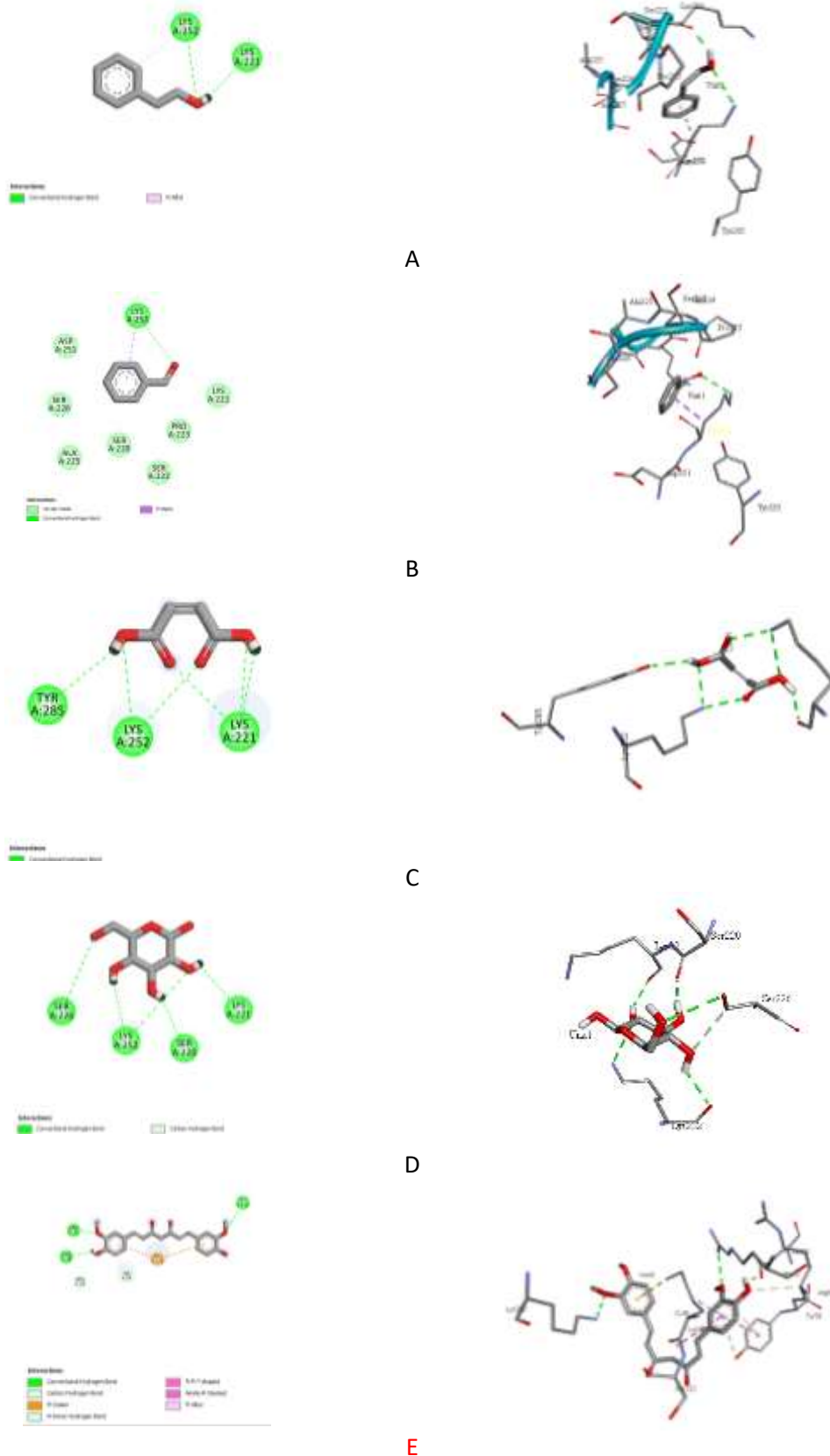
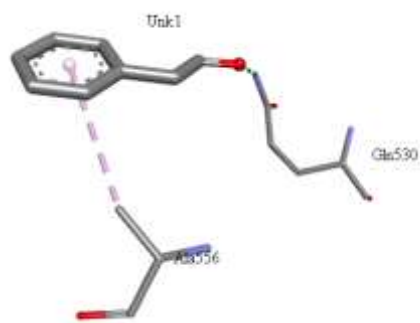
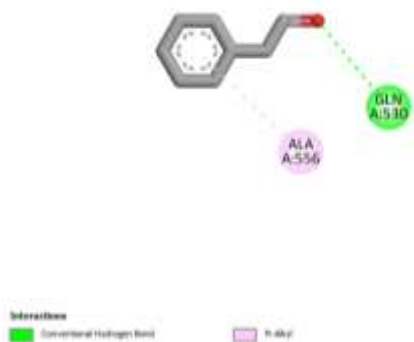
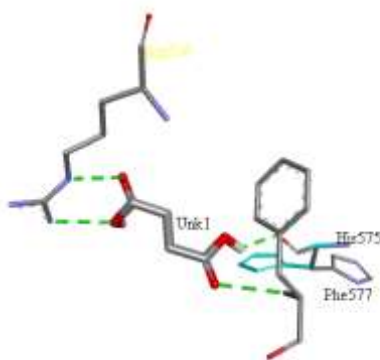
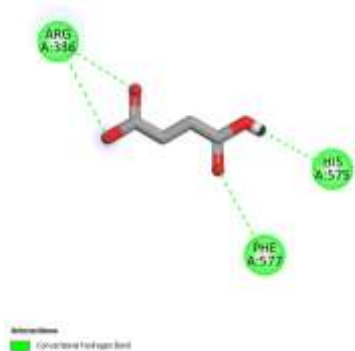


Fig. 4. 2D and 3D representation of the kind of interaction on the formation of the NF-κB active site (PDB ID 1A3Q). A) 2-Phenylethanol, B) Benzaldehyde, C) Fumaric acid, D) Glucose, and E) Curcumin.

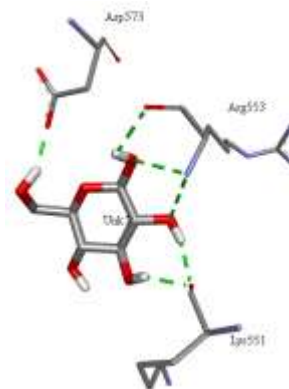
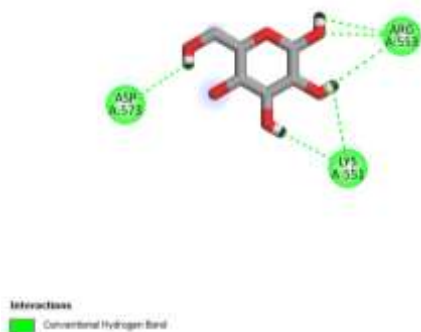
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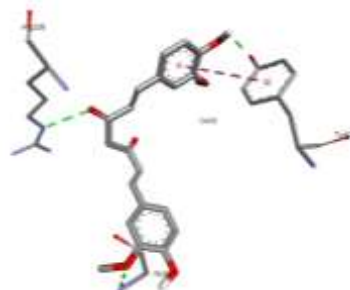
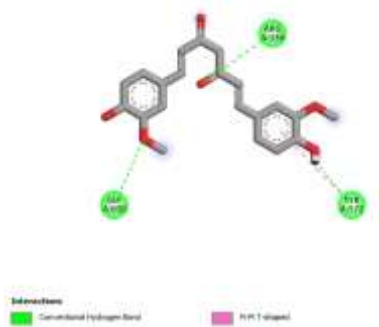
A



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.

259

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

269 Curcumin (the reference compound) created three hydrogen bonds with the amino acid
270 residues ARG52, GLU58, and LYS252 to interact with the active site of NF-κB. Meanwhile,
271 curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572,
272 and GLY600 to interact with the active site of nrf2 Keap1 (Figs 4 and 5). Despite the fact that
273 curcumin could create three hydrogen bonds at both the active sites of NF-κB and nrf2 Keap1,
274 its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol,
275 benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to
276 interact with NF-κB and nrf2 Keap1 receptors. Whereas, fumaric acid and 2-
277 phenylacetaldehyde were metabolites that had the strongest interaction with NF-κB (PDB ID
278 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

279 DISCUSSION

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

288 Carbon tetrachloride (CCl₄) is a component of the hepatotoxin, which acts after metabolic
289 activation and is extensively employed as a liver-damaging agent. In this study, the
290 administration of CCl₄ to animals caused hepatotoxicity, which was characterized by a
291 significant increase in ALT after several administrations. CCl₄ is metabolized by the enzyme
292 cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl
293 radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl
294 peroxy radical, which rapidly reacts with lipids to form lipid peroxidation products. Free
295 radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased
296 protein synthesis, and increased oxidase activity. CCl₄ hepatotoxicity is characterized by
297 hepatocellular necrosis with fat deposition (Ritesh et al. 2015). At the molecular level,
298 administration of CCl₄ can activate tumor necrosis factor (TNF)-α, nitric oxide (NO), and
299 transforming growth factor (TGF)-α and -β in cells, processes that precipitate cell self-
300 destruction or fibrosis. TNF-α leads to apoptosis, whereas TGF-β leads to fibrosis (Weber et al.
301 2003).

302 In terms of its pathophysiological underpinnings, liver illness is linked to a condition known
303 as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Zavala et al.
304 2016; Laureys and De Vuyst 2017; Romero-Luna et al. 2020). Both qualitative and quantitative
305 changes in the gut microbiome have the potential to affect the composition of products
306 produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et al.

307 2020). Qualitative changes include an imbalance between harmful and helpful microbiomes,
308 whereas numeric changes involve changes to the overall microbiota. In addition to these
309 symptoms, intestinal inflammation, disruption of the intestinal barrier, and the transfer of
310 microbial products can all be caused by dysbiosis (Laureys et al. 2018). For this reason, the
311 condition of the gut microbiome is an important factor in the initiation and development of
312 chronic liver disease (Lee et al. 2021). Based on the results of the study, treatment with water
313 kefir for 2 weeks after the occurrence of liver damage was able to improve the overall condition
314 of the liver which was marked by a significant decrease in the values of AST, ALT, TNF- α ,
315 TGF- β , and significant improvement in liver histology.

316 Water kefir contains a number of microorganisms that have been linked to health benefits,
317 such as the probiotics *L. paracasei* and *B. cereus* (Fijan 2014). This activity is linked to an increase
318 in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription
319 factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4).
320 Improvements in intestinal barrier function and histological integrity were also observed.
321 Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs
322 simultaneously with the restoration of the p38 MAPK pathway (Fijan 2014; Yao et al. 2019;
323 Tsai et al. 2020; Ji et al. 2022). *Bacillus* is a kind of endospore-forming bacterium that can
324 endure extremely cold temperatures and lengthy periods of storage without dying; its spores
325 can even tolerate the acidic environment of the stomach and make it all the way to the small
326 intestine (Elshagabee et al. 2017). *B. cereus* has been shown to reduce ALT levels, an indicator
327 of liver healing, in various animal models of liver injury. It protects the liver by reducing
328 inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines
329 (Kim et al. 2018; Li et al. 2019a; Xue et al. 2020). Also, when *Bacillus* spores were used first,
330 hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This
331 effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al.
332 2020).

333 Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-
334 negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria
335 apart from others is their alkaline-stable lipid membrane (Lynch et al. 2021). Their "oxidative"
336 fermentation metabolism is responsible for the principal metabolic process in these bacteria,
337 the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond,
338 fermentation helps mediate the transition of diverse substrates into products. Although lactic
339 acid bacteria have been studied more extensively than acetic acid bacteria (Semjonovs et al.
340 2014; Hong et al. 2021), various studies have shown promising results concerning the
341 pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic
342 acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the
343 severity of liver injury in rats with septic shock by increasing the expression of TRIM40.
344 TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of
345 cytokines such as IL-6 and TNF- α , raise the expression of IL-10, improve survival in septic
346 mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased
347 inflammation as well as the production of inflammatory cytokines (Yang et al. 2019). Acetic
348 acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation
349 and reduced hepatic lipid and body fat deposition (Kondo et al. 2009; Li et al. 2018).

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

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

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

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

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

401 In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF- κ B plays a
402 crucial role in regulating inflammation and cell death (Luedde and Schwabe 2011). In response
403 to many stimuli that may pose a threat to the host, NF- κ B is activated, setting in motion
404 processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde
405 and Schwabe 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs)
406 include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory
407 cytokines including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe
408 2011). When NF- κ B is activated, a lot of genes with B-binding sites are transcribed. These genes
409 play important roles in controlling inflammation, the immune response, and cell survival. In
410 an NF- κ B-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by
411 reducing BAMBI expression (an inhibitory TGF- β pseudoreceptor) and increasing Kupffer cell
412 chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF- β , which
413 stimulates HSCs unrestrictively. When HSCs have been activated, NF- κ B serves a second
414 crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators
415 (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine
416 way, all play roles in activating NF- κ B in activated hepatic stellate cells. More activated HSCs
417 and extracellular matrix are deposited in the liver as a result of greater HSC activation and
418 survival (Luedde and Schwabe 2011).

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

427 CONCLUSION

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

435

436 CONFLICT OF INTEREST

437 The authors declare no conflict of interest.

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- 676

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	<ol style="list-style-type: none">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.	<ol style="list-style-type: none">1. We have added the research problem2. We have revised the last sentence
MATERIAL AND METHODS	<ol style="list-style-type: none">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-α 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	<ol style="list-style-type: none"> 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 :	<ol style="list-style-type: none"> 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.openaccessjournal.com/blog/predatory-journals-list/#l_%E2%80%93predatory_journals). References must be write free of codes from any bibliography processor, such as Endnotes, Mendeley, Zotero, etc...2. Latest references must be used. 	<ol style="list-style-type: none"> 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 CCl₄-induced liver injury in rats

Running title: Hepatoprotective study of water kefir

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19 **Contribution Details** (to be ticked marked (X) as applicable and add columns according to the number of
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21

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

22

23 ABSTRACT

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

27 Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl₄-induced acute liver injury and to in silico
28 investigate metabolites that play an important role in hepatoprotective mechanisms.

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

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

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

41
42 Keywords: free radicals; liver diseases; kefir; molecular docking simulation; probiotics

43 44 INTRODUCTION

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

57 Water kefir has been used medicinally for a very long time, and recent research has
58 indicated that it may have a variety of positive effects on people's health. It has been
59 demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the
60 production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*,
61 *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus*
62 *ochraceus*, *A. niger*, *A. flavus*, *Penicillium sp.*, and *Rhizopus sp.* (Al-Mohammadi et al., 2021). In
63 addition to its antibacterial properties, water kefir possesses a broad spectrum of
64 pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al.,
65 2003; Aligita et al., 2020), antioxidant (Alsayadi et al., 2013; Aligita et al., 2020; Darvishzadeh
66 et al., 2021), hepatoprotective (Aspiras et al., 2015; Aligita et al., 2021), antihyperglycemic and
67 antihyperlipidemic (Alsayadi et al., 2014; Rocha-Gomes et al., 2018), anti-edematous (Moreira
68 et al., 2008), antitumor (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019),
69 immunomodulant (Calatayud et al., 2021), and anti-ulcerogenic (Rodrigues et al., 2016).
70 However, no studies have been reported on the hepatoprotective effects of water kefir against
71 carbon tetrachloride (CCl₄)-induced liver injury.

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

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

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

100 Water kefir has been used for an extensive period of time and has been recognized for its
101 widespread benefits, especially in Indonesia. However, its level of popularity falls short in
102 comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused
103 on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the
104 purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl₄-
105 induced rats while also investigating the stability interactions of its metabolites within the NF-
106 κB and Nrf2 receptors using molecular docking studies.

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

109 MATERIAL AND METHODS

110 Materials and reagents

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

115 Experimental sample and reference extract

116 The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution
117 was produced using a fermentation procedure. The initial stage involved the preparation of
118 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The
119 sugar and warm distilled water were mixed in a beaker, followed by the addition of water
120 kefir grains and raisins to the resulting sugar solution. The fermentation procedure was
121 conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

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

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

128 **Animals and experimental design**

129 Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water
130 *ad libitum*. Four mice in each group were used. All procedures relating to animals and their
131 care conformed to the international guidelines Principles of Laboratory Animal Care (NIH
132 publication no. 85-23, revised 1985) with the ethical approval number
133 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats
134 received CCl₄ (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al.
135 2011). The rats were randomized into five groups after the development of animals with liver
136 injury, which is characterized by a significant increase in serum ALT level, as follows: (1)
137 positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg
138 BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with
139 the addition (6) negative control group. Each group received group-specific treatment for two
140 weeks, along with the administration of 1.25 mL/kg BW of CCl₄ (20% in olive oil) every three
141 days.

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

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

155 **Molecular docking simulation**

156 Molecular docking experiments were done with the PyRx software (Dallakyan and Olson
157 2015) to predict how metabolites, which are small-molecule ligands, bind to biological
158 macromolecules. The NCBI PubChem database (<https://pubchem.nlm.nih.gov/>, accessed on
159 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites
160 (Patel et al. 2022). Target proteins like NF- κ B (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B)
161 were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/>, accessed on 03 May
162 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et
163 al. 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study
164 (Febrina et al. 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the
165 protein and ligand complex and distance (Ischak et al. 2023). The BIOVIA Discovery Studio
166 2017 R2 tool was also utilized to find protein active sites.

167 **Statistical analysis**

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

174 **RESULTS**

175 ***In vivo* evaluation of hepatoprotective activity**

176 The serum ALT levels, as the main parameter for the liver damage, were measured prior to
 177 and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment
 178 AST, TNF- α , and TGF- β levels were also evaluated. These findings were analyzed using a one-
 179 way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant
 180 rise in blood ALT levels demonstrated the establishment of an animal model with liver injury,
 181 according to statistical analysis, following the administration of CCl₄. When compared to the
 182 positive control group, ALT serum levels decreased significantly after two weeks of therapy
 183 with curcuma extract or water kefir. The three doses of water kefir groups demonstrated
 184 equivalent activity when curcuma extract was used as the standard treatment, and there was
 185 no significant difference between the three doses of water kefir. When compared to the
 186 positive control group, AST levels were also reduced dramatically following treatment with
 187 curcuma extract or water kefir. TNF- α levels in the water kefir group were significantly lower
 188 than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though
 189 there was no statistically significant difference in TGF- β levels, the group that received the
 190 treatment demonstrated a decrease in TGF- β levels.

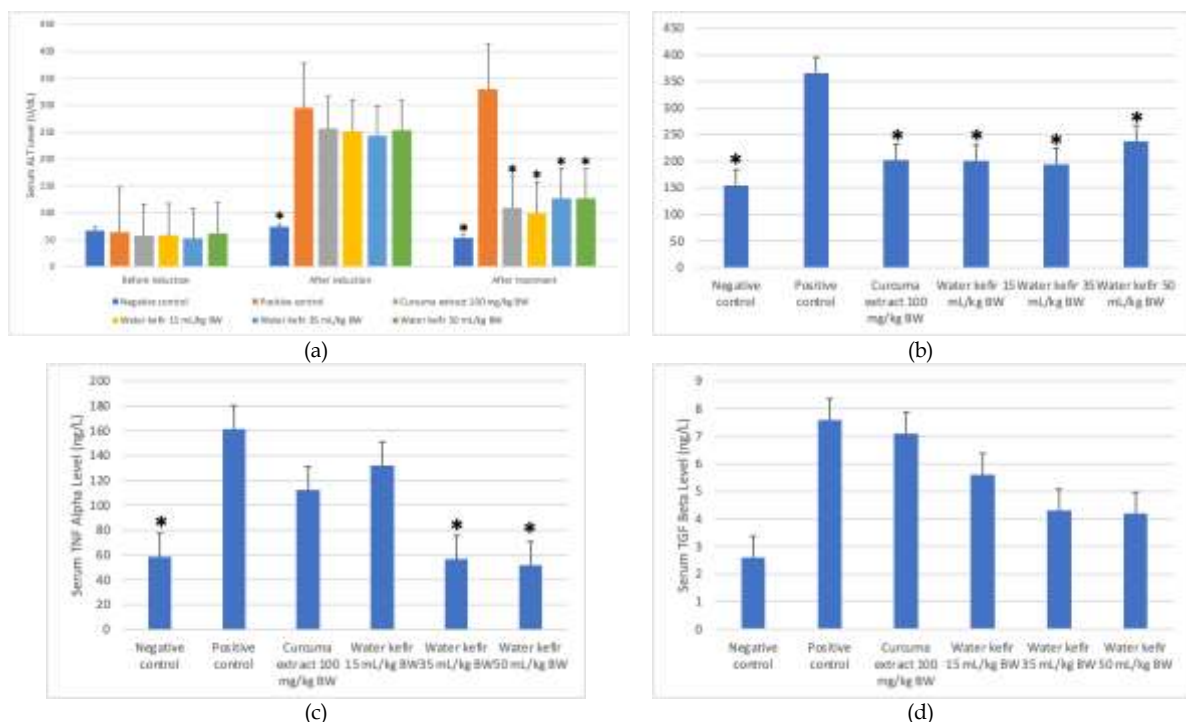


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF- α level; (d) After treatment serum TGF- β level. * indicates a significantly different result when compared to the positive control group; # indicates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

192 A histological examination of a normal liver group revealed a typical central vein bordered
 193 with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct
 194 cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl₄-induced group developed
 195 centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and
 196 necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The
 197 group that received either curcuma extract or water kefir treatment improved in varied
 198 necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2
 199 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir
 200 30 mL/kg BW (Fig 2I & 2J)).

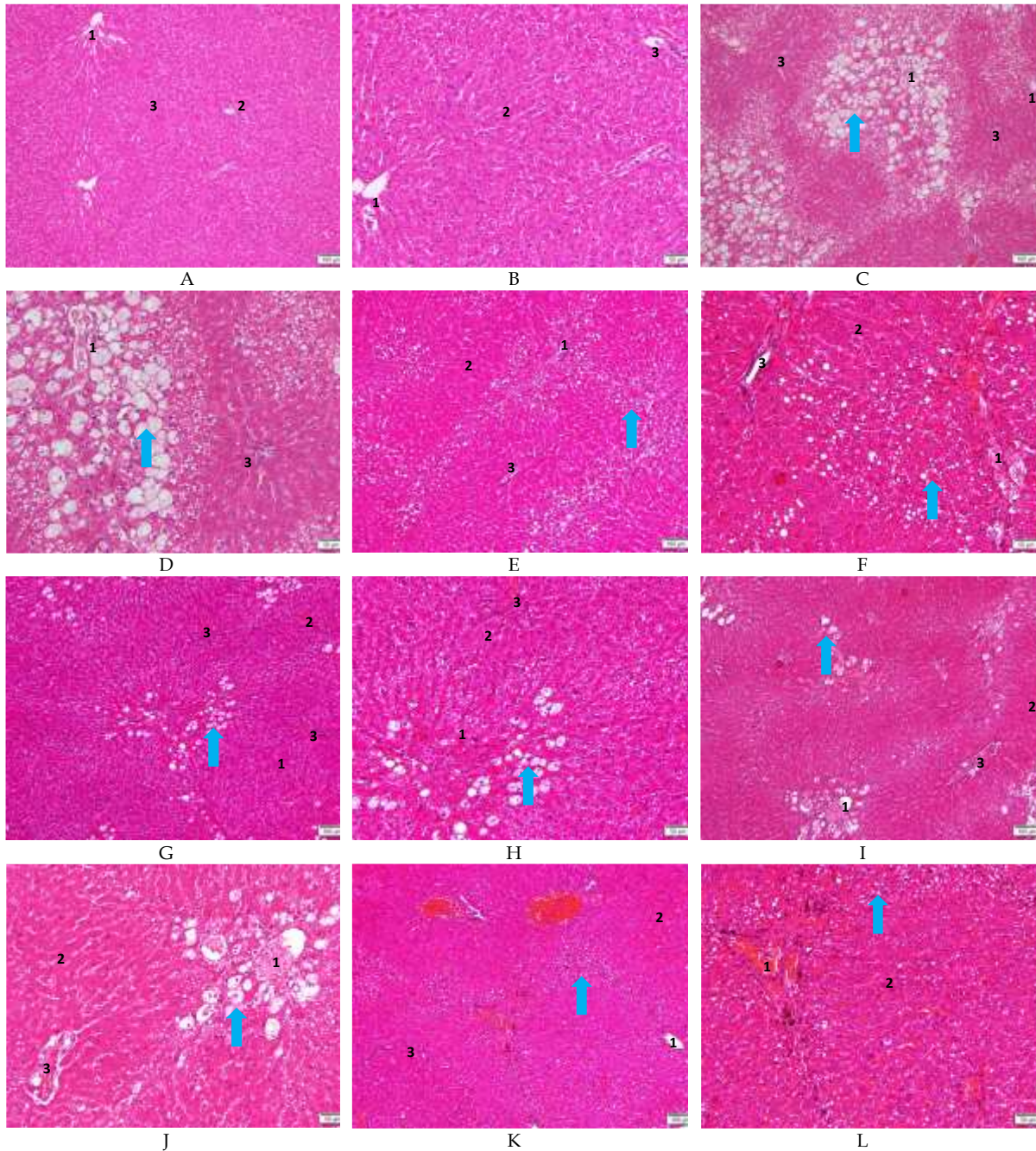


Fig. 2. Liver histology after CCl₄ intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal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

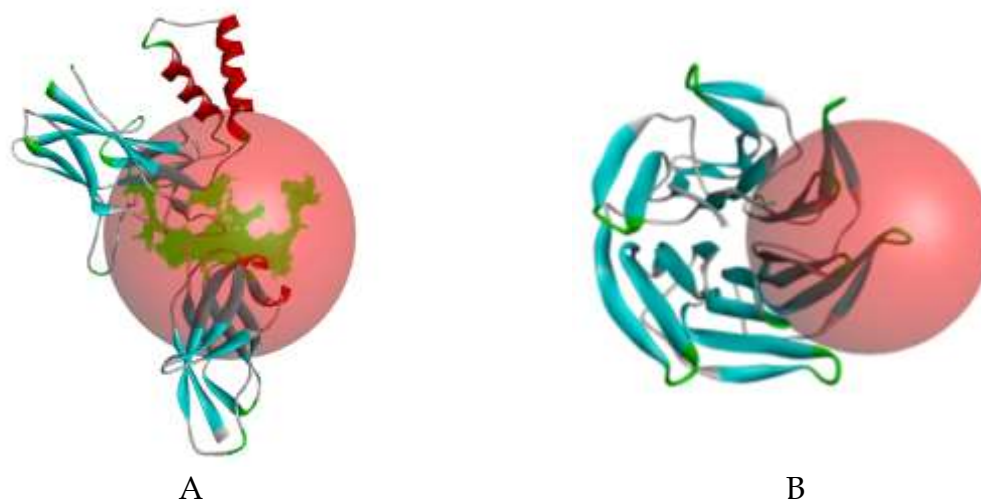


Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF- κ B (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

217

218 The docking results of the 25 metabolites could interact with target proteins (Keap1 and
219 NF- κ B) (Table 1). In general, all metabolites could interact with both NF- κ B 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- κ B 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- κ B 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

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

237

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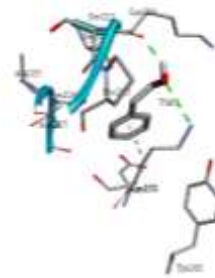
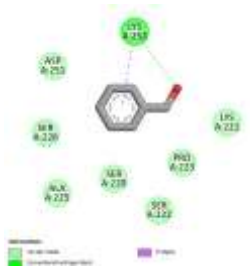
239 **Table 1.** The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF-
 240 κ B (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B)

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

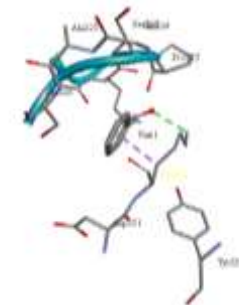
241

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

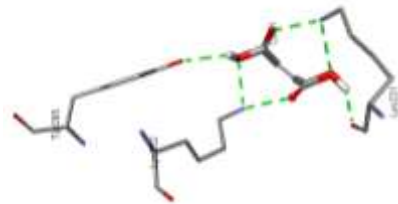
254 in the interaction, so fumaric acid interacted more strongly with the active site of NF- κ B (Fig.
 255 4).
 256



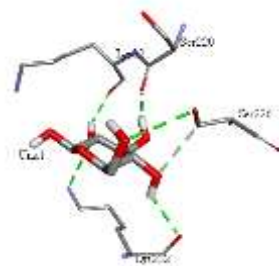
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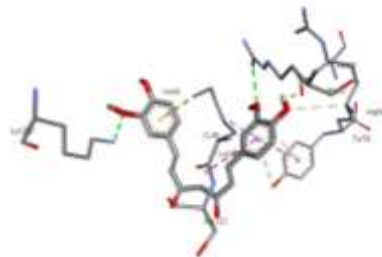
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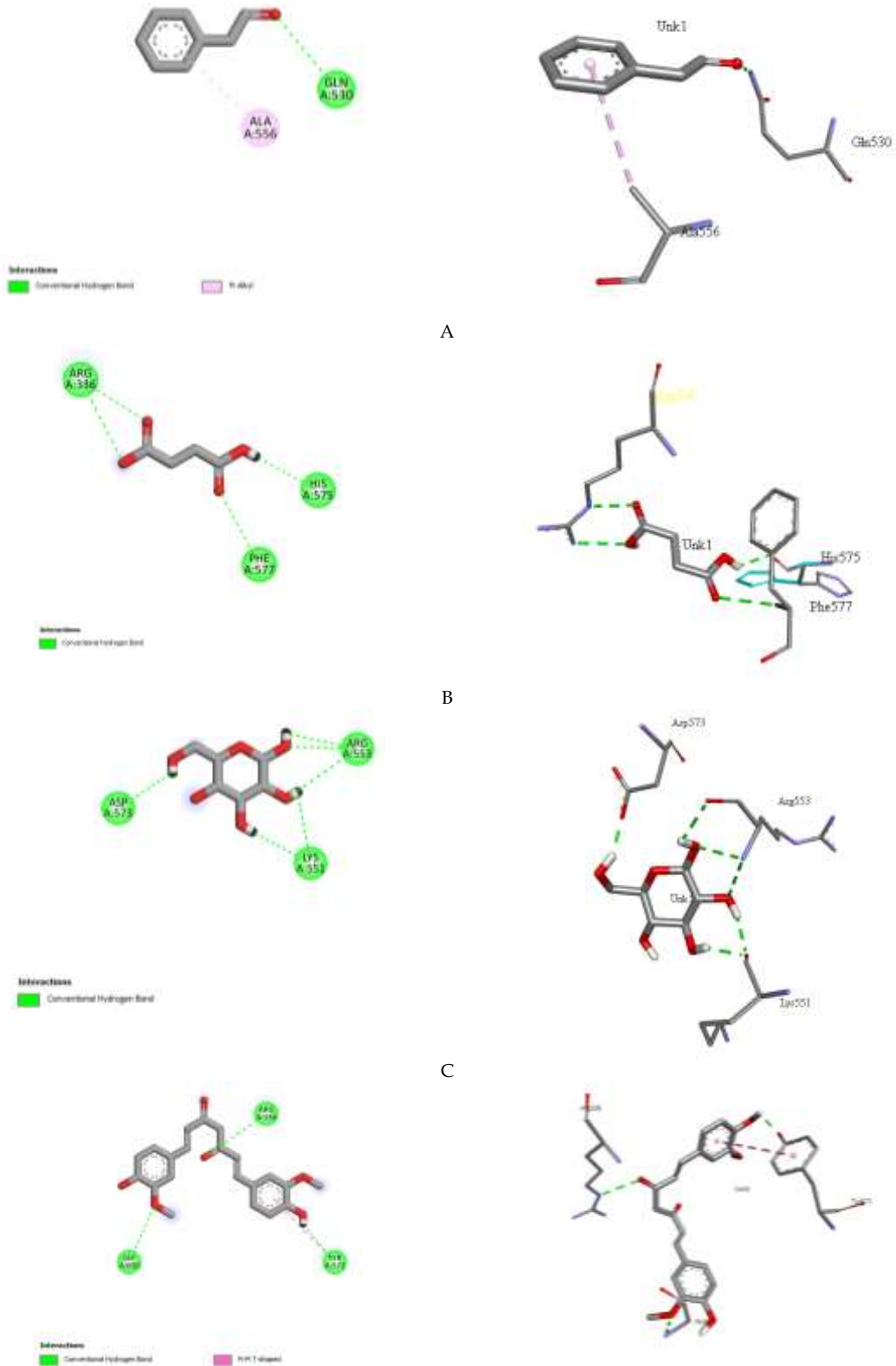
E

Insert here the figures corresponding to curcumin.

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

257

258



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.

259

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

269 Curcumin (the reference compound) created three hydrogen bonds with the amino acid
270 residues ARG52, GLU58, and LYS252 to interact with the active site of NF- κ B. Meanwhile,
271 curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572,
272 and GLY600 to interact with the active site of nrf2 Keap1 (Figs. 4 and 5). Despite the fact that
273 curcumin could create three hydrogen bonds at both the active sites of NF- κ B and nrf2 Keap1,
274 its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol,
275 benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to
276 interact with NF- κ B and nrf2 Keap1 receptors. Whereas fumaric acid and 2-
277 phenylacetaldehyde were metabolites that had the strongest interaction with NF- κ B (PDB ID
278 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

279

280 DISCUSSION

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

289 Carbon tetrachloride (CCl₄) is a component of the hepatotoxin, which acts after metabolic
290 activation and is extensively employed as a liver-damaging agent. In this study, the
291 administration of CCl₄ to animals caused hepatotoxicity, which was characterized by a
292 significant increase in ALT after several administrations. CCl₄ is metabolized by the enzyme
293 cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl
294 radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl
295 peroxy radical, which rapidly reacts with lipids to form lipid peroxidation products. Free
296 radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased
297 protein synthesis, and increased oxidase activity. CCl₄ hepatotoxicity is characterized by
298 hepatocellular necrosis with fat deposition (Ritesh et al. 2015). At the molecular level,
299 administration of CCl₄ can activate tumor necrosis factor (TNF)- α , nitric oxide (NO), and
300 transforming growth factor (TGF)- α and - β in cells, processes that precipitate cell self-
301 destruction or fibrosis. TNF- α leads to apoptosis, whereas TGF- β leads to fibrosis (Weber et
302 al., 2003).

303 In terms of its pathophysiological underpinnings, liver illness is linked to a condition known
304 as dysbiosis, which refers to an imbalance in the makeup of the gut microbiota (Zavala et al.,
305 2016; Laureys and De Vuyst, 2017; Romero-Luna et al., 2020). Both qualitative and quantitative
306 changes in the gut microbiome have the potential to affect the composition of products
307 produced by the microbiota, such as short-chain fatty acids and bile acids (Romero-Luna et
308 al., 2020). Qualitative changes include an imbalance between harmful and helpful
309 microbiomes, whereas numeric changes involve changes to the overall microbiota. In addition
310 to these symptoms, intestinal inflammation, disruption of the intestinal barrier, and the
311 transfer of microbial products can all be caused by dysbiosis (Laureys et al., 2018). For this
312 reason, the condition of the gut microbiome is an important factor in the initiation and
313 development of chronic liver disease (Lee et al., 2021). Based on the results of the study,
314 treatment with water kefir for 2 weeks after the occurrence of liver damage was able to
315 improve the overall condition of the liver, which was marked by a significant decrease in the
316 values of AST, ALT, TNF- α , TGF- β , and significant improvement in liver histology.

317 Water kefir contains a number of microorganisms that have been linked to health benefits,
318 such as the probiotics *L. paracasei* and *B. cereus* (Fijan 2014). This activity is linked to an increase
319 in antioxidants like glutathione and catalase and a decrease in pro-inflammatory transcription
320 factors like nuclear B-factor, lipopolysaccharide, and Toll-like receptor 4 (TLR-4).
321 Improvements in intestinal barrier function and histological integrity were also observed.
322 Increased expression of claudin-1 and occludin-1, two components of tight junctions, occurs
323 simultaneously with the restoration of the p38 MAPK pathway (Fijan 2014; Yao et al. 2019;
324 Tsai et al. 2020; Ji et al. 2022). *Bacillus* is a kind of endospore-forming bacterium that can
325 endure extremely cold temperatures and lengthy periods of storage without dying; its spores
326 can even tolerate the acidic environment of the stomach and make it all the way to the small
327 intestine (Elshagabee et al. 2017). *B. cereus* has been shown to reduce ALT levels, an indicator
328 of liver healing, in various animal models of liver injury. It protects the liver by reducing
329 inflammation, enhancing the gut flora, and strengthening the tight junctions in the intestines
330 (Kim et al. 2018; Li et al. 2019a; Xue et al. 2020). Also, when *Bacillus* spores were used first,
331 hepatocyte necrosis and serum levels of ALT, ZO-1, AST, and TAC went down by a lot. This
332 effect is comparable to that of the popular hepatoprotective compound silymarin (Neag et al.
333 2020).

334 Beer, vinegar, cider, and cocoa bean masses are just some of the places where Gram-
335 negative acetic acid bacteria have been found. One of the features that sets acetic acid bacteria
336 apart from others is their alkaline-stable lipid membrane (Lynch et al. 2021). Their "oxidative"
337 fermentation metabolism is responsible for the principal metabolic process in these bacteria,
338 the oxidation of ethanol to acetic acid. Important in the food and beverage sector and beyond,
339 fermentation helps mediate the transition of diverse substrates into products. Although lactic
340 acid bacteria have been studied more extensively than acetic acid bacteria (Semjonovs et al.
341 2014; Hong et al. 2021), various studies have shown promising results concerning the
342 pharmacological effects of acetic acid bacteria, especially as a hepatoprotective agent. Acetic
343 acid, which is the main metabolite of acetic acid bacteria, can lower inflammation and the
344 severity of liver injury in rats with septic shock by increasing the expression of TRIM40.
345 TRIM40 has been shown to minimize liver damage, decrease the synthesis and release of
346 cytokines such as IL-6 and TNF- α , raise the expression of IL-10, improve survival in septic
347 mice, and block the activity of the TLR4 signaling pathway. Acetic acid injection decreased
348 inflammation as well as the production of inflammatory cytokines (Yang et al. 2019). Acetic
349 acid also stimulates the AMPK signaling pathway, which leads to enhanced lipid oxidation
350 and reduced hepatic lipid and body fat deposition (Kondo et al. 2009; Li et al. 2018).

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

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

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

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

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

402 In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF- κ B plays a
403 crucial role in regulating inflammation and cell death (Luedde and Schwabe 2011). In response
404 to many stimuli that may pose a threat to the host, NF- κ B is activated, setting in motion
405 processes such as inflammation, immunity, wound healing, and pathogen clearance (Luedde
406 and Schwabe 2011). Pathogen-derived chemicals that activate Toll-Like Receptors (TLRs)
407 include lipopolysaccharide (LPS), viral and bacterial DNA and RNA, and inflammatory
408 cytokines, including tumor necrosis factor (TNF) and interleukins (IL)-1 (Luedde and Schwabe
409 2011). When NF- κ B is activated, a lot of genes with B-binding sites are transcribed. These genes
410 play important roles in controlling inflammation, the immune response, and cell survival. In
411 an NF- κ B-dependent manner, lipopolysaccharide (LPS) activates TLR4 on dormant HSCs by
412 reducing BAMBI expression (an inhibitory TGF- β pseudoreceptor) and increasing Kupffer cell
413 chemotaxis. Due to low levels of BAMBI, recruited Kupffer cells secrete TGF- β , which
414 stimulates HSCs unrestrictively. When HSCs have been activated, NF- κ B serves a second
415 crucial function by increasing their chances of survival. LPS, Kupffer cell-derived mediators
416 (such as IL-1 and TNF), and angiotensin II, which is produced and acts on HSCs in an autocrine
417 way, all play roles in activating NF- κ B in activated hepatic stellate cells. More activated HSCs
418 and extracellular matrix are deposited in the liver as a result of greater HSC activation and
419 survival (Luedde and Schwabe 2011).

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

428 CONCLUSION

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

436

437 CONFLICT OF INTEREST

438 The authors declare no conflict of interest.

439 ACKNOWLEDGMENT

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441 Bandung, West Java, Republic of Indonesia (052/14.LPPM/PE.I/LPPM/2021)

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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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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

22

23 ABSTRACT

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

27 Objectives: To evaluate the efficacy of water kefir in vivo against hepatoprotective CCl₄-induced acute liver injury and to in silico
28 investigate metabolites that play an important role in hepatoprotective mechanisms.

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

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

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

41
42 Keywords: free radicals; liver diseases; kefir; molecular docking simulation; probiotics

43 INTRODUCTION

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

57 Water kefir has been used medicinally for a very long time, and recent research has
58 indicated that it may have a variety of positive effects on people's health. It has been
59 demonstrated that water kefir contains non-pathogenic bacteria that, in conjunction with the
60 production of organic acids, can inhibit the growth of pathogenic microbes such as *Shigella sp.*,
61 *Salmonella sp.*, *Staphylococcus aureus*, and *E. coli*; as well as, filamentous fungi such as *Aspergillus*
62 *ochraceus*, *A. niger*, *A. flavus*, *Penicillium sp.*, and *Rhizopus sp.* (Al-Mohammadi et al., 2021). In
63 addition to its antibacterial properties, water kefir possesses a broad spectrum of
64 pharmacological effects. Some of these therapeutic effects are anti-inflammatory (Diniz et al.,
65 2003; Aligita et al., 2020), antioxidant (Aligita et al., 2020; Darvishzadeh et al., 2021),
66 hepatoprotective (Aspiras et al., 2015; Aligita et al., 2021), antihyperglycemic and
67 antihyperlipidemic (Alsayadi et al., 2014; Rocha-Gomes et al., 2018), anti-edematous (Moreira
68 et al., 2008), antitumor (Zamberi et al., 2016), antihypertensive (Gamba et al., 2019),
69 immunomodulant (Calatayud et al., 2021), and anti-ulcerogenic (Rodrigues et al., 2016).
70 However, no studies have been reported on the hepatoprotective effects of water kefir against
71 carbon tetrachloride (CCl₄)-induced liver injury.

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

76 2-related factor 2 (Nrf2) pathways, respectively. It has been shown that activating Nrf2 and
77 inhibiting NF- κ B can reduce the amount of damage done to the liver. For instance, curcumin
78 protects against aflatoxin B1-induced liver injury by increasing the expression of Nrf2 and
79 related downstream antioxidant molecules (such as superoxide dismutase (SOD), catalase
80 (CAT), heme oxygenase 1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO-1) (Wang
81 et al., 2022b). Also, it has been shown that the molecule methoxy eugenol, which comes from
82 nutmeg and Brazilian red propolis, protects the liver both *in vitro* and *in vivo*. This may be
83 attributed to the fact that it targets the NF- κ B signaling pathway, which has been shown to
84 have anti-inflammatory effects (De Souza Basso et al., 2021).

85 Ethanol and lactic acid are the primary metabolites found in fermented water kefir, while
86 lower quantities of glycerol, mannitol, and acetic acid can also be found in the beverage.
87 Additionally, a variety of aromatic and volatile compounds are produced, including ethyl
88 acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, and ethyl decanoate, among others
89 (compared to their threshold levels) (Laureys and De Vuyst 2014). The chemical constituents
90 of both phytochemicals and secondary metabolites in natural products, including water kefir,
91 are certainly capable of providing various pharmacological effects for the body (Asnawi et al.,
92 2022; Nursamsiar et al., 2022). However, an *in silico* study to evaluate the metabolite content
93 in water kefir has not been reported yet. Because of its capacity to speed up the process of
94 identifying and optimizing lead compounds, the *in silico* method has become the front-runner
95 in the race to improve the speed and accuracy of the process of discovering new drugs. This is
96 because the *in silico* method can identify and optimize lead compounds more quickly.
97 Techniques such as molecular docking and molecular dynamics (MD) were able to directly
98 indicate a small number of compounds that have high affinity and selectivity by analyzing
99 how the ligand and target interact with one another (Febrina et al., 2021).

100 Water kefir has been used for an extensive period of time and has been recognized for its
101 widespread benefits, especially in Indonesia. However, its level of popularity falls short in
102 comparison to that of milk kefir. Furthermore, there is a scarcity of studies specifically focused
103 on the hepatoprotective activity of water kefir made with Indonesian grains. Therefore, the
104 purpose of this study was to evaluate the hepatoprotective effects of water kefir in CCl₄-
105 induced rats while also investigating the stability interactions of its metabolites within the NF-
106 κ B and Nrf2 receptors using molecular docking studies.

107

108 MATERIAL AND METHODS

109 Materials and reagents

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

114 Experimental sample and reference extract

115 The water kefir grain was obtained from Yogyakarta, Indonesia. The water kefir solution
116 was produced using a fermentation procedure. The initial stage involved the preparation of
117 60 g of sugar, 2 g of raisins, 100 g of water kefir grains, and 1 L of aqua mineral distillate. The
118 sugar and warm distilled water were mixed in a beaker, followed by the addition of water
119 kefir grains and raisins to the resulting sugar solution. The fermentation procedure was
120 conducted over a duration of forty-eight hours at ambient temperature. A small cloth was used

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

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

127 **Animals and experimental design**

128 Rats (Wistar strain, male, 200–250 g) were maintained on normal pellet food and tap water
129 *ad libitum*. Four mice in each group were used. All procedures relating to animals and their
130 care conformed to the international guidelines Principles of Laboratory Animal Care (NIH
131 publication no. 85-23, revised 1985) with the ethical approval number
132 363/UN6.KEP/EC/2020. In order to develop an animal model with liver injury, the rats
133 received CCl₄ (20% in olive oil) at 1.25 mL/kg BW every 2 days via a gavage tube (Yeh et al.
134 2011). The rats were randomized into five groups after the development of animals with liver
135 injury, which is characterized by a significant increase in serum ALT level, as follows: (1)
136 positive control group, (2) Curcuma extract group 100 mg/kg BW, (3) water kefir 15 mL/kg
137 BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with
138 the addition (6) negative control group. Each group received group-specific treatment for two
139 weeks, along with the administration of 1.25 mL/kg BW of CCl₄ (20% in olive oil) every three
140 days.

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

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

154 **Molecular docking simulation**

155 Molecular docking experiments were done with the PyRx software (Dallakyan and Olson,
156 2015) to predict how metabolites, which are small-molecule ligands, bind to biological
157 macromolecules. The NCBI PubChem database (<https://pubchem.nlm.nih.gov/>, accessed on
158 3 May 2023) was used to derive the three-dimensional structure of water kefir metabolites
159 (Patel et al., 2022). Target proteins like NF- κ B (PDB code: 1A3Q) and Keap1 (PDB code: 4L7B)
160 were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/>, accessed on 03 May
161 2023). The PyRx program was used to reduce protein, ligand converted to PDBQT (Asnawi et
162 al., 2023), then maximize GRID parameter (Asnawi et al. 2022) and perform docking study
163 (Febrina et al., 2022). The BIOVIA Discovery Studio 2017 R2 program was used to view the
164 protein and ligand complex and distance (Ischak et al., 2023). The BIOVIA Discovery Studio
165 2017 R2 tool was also utilized to find protein active sites.

166 **Statistical analysis**

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

173 **RESULTS**

174 ***In vivo* evaluation of hepatoprotective activity**

175 The serum ALT levels, as the main parameter for the liver damage, were measured prior to
 176 and following induction, as well as following treatment (Fig. 1). Meanwhile, after treatment
 177 AST, TNF- α , and TGF- β levels were also evaluated. These findings were analyzed using a one-
 178 way ANOVA with a 95% confidence level and a two-sided confidence interval. A significant
 179 rise in blood ALT levels demonstrated the establishment of an animal model with liver injury,
 180 according to statistical analysis, following the administration of CCl₄. When compared to the
 181 positive control group, ALT serum levels decreased significantly after two weeks of therapy
 182 with curcuma extract or water kefir. The three doses of water kefir groups demonstrated
 183 equivalent activity when curcuma extract was used as the standard treatment, and there was
 184 no significant difference between the three doses of water kefir. When compared to the
 185 positive control group, AST levels were also reduced dramatically following treatment with
 186 curcuma extract or water kefir. TNF- α levels in the water kefir group were significantly lower
 187 than in the positive control group at dosages of 35 and 50 mL/kg body weight. Even though
 188 there was no statistically significant difference in TGF- β levels, the group that received the
 189 treatment demonstrated a decrease in TGF- β levels.

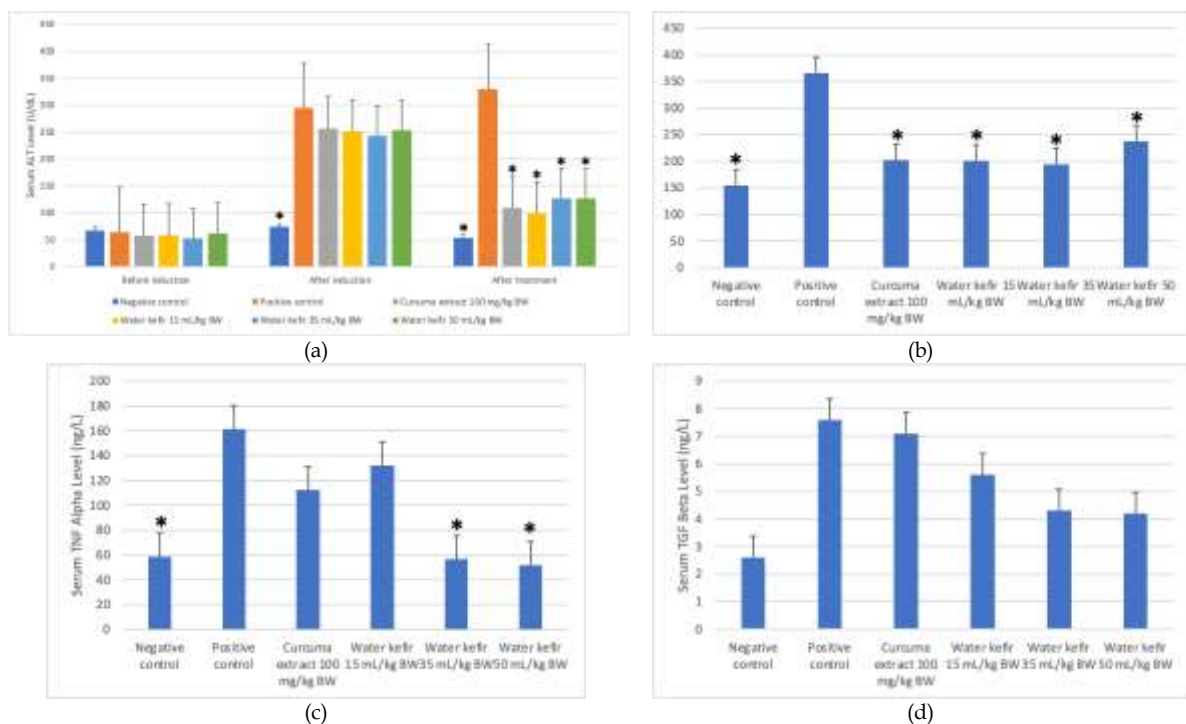


Fig. 1. (a) Before induction, after induction, and after treatment serum ALT level; (b) After treatment serum AST level; (c) After treatment serum TNF- α level; (d) After treatment serum TGF- β level. * indicates a significantly different result when compared to the positive control group; # indicates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

191 A histological examination of a normal liver group revealed a typical central vein bordered
 192 with endothelial cells and normal hepatocytes, resulting in hepatic cords of cells with distinct
 193 cell borders and sinusoidal gaps (Figs 2A and 2B). The CCl₄-induced group developed
 194 centrilobular hepatic necrosis level 4, which was characterized by massive vacuolization and
 195 necrosis of epithelial cells in the liver's centrilobular region (blue arrow) (Fig 2C & 2D). The
 196 group that received either curcuma extract or water kefir treatment improved in varied
 197 necrotic conditions ranging from level 1 (water kefir 50 mL/kg BW) (Fig 2K & 2L) to level 2
 198 (curcuma extract (Fig 2E & 2F), water kefir 15 mL/kg BW (Figs 2G and 2H), and water kefir
 199 30 mL/kg BW (Fig 2I & 2J)).

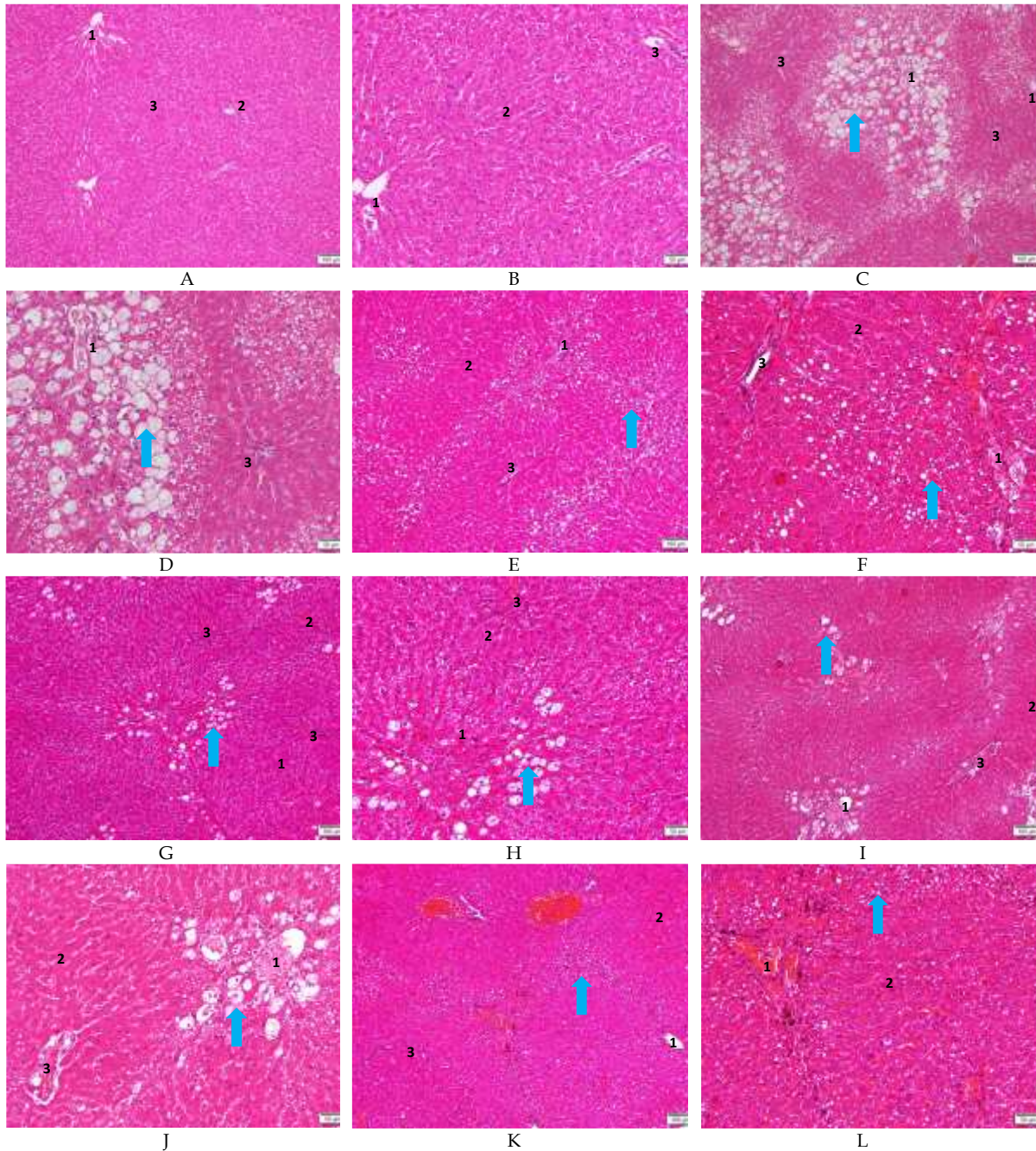


Fig. 2. Liver histology after CCl₄ intoxication and treatment with HE staining. Negative control group (A & B), positive control group (C & D), curcuma extract group (E & F), water kefir 15 mL/kg BW (G & H), water kefir 30 mL/kg BW (I & J), and water kefir 50 mL/kg BW (K & L). 1: central vein; 2: normal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

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Molecular docking

204 Molecular docking studies are considered a powerful tool for predicting the potential
205 targets of bioactive molecules. In order to carry out molecular docking simulations, one of the
206 most critical steps is to identify the target active site. If the target protein is crystallized with a
207 native ligand, in many instances, the location of the active site can be established without any
208 difficulty (Li et al., 2019b). However, the NF- κ B (PDB ID 1A3Q) and nrf2 Keap1 (PDB ID 4L7B)
209 proteins do not have a native ligand, so the active site was determined. Active site prediction
210 in docking is a computational method for predicting the location and orientation of a receptor
211 protein's binding site for a ligand molecule. The active site prediction was based on a protein
212 structural analysis and the identification of amino acid residues that are likely to interact with
213 the ligand. The projected binding site is then utilized as a starting point for molecular docking,
214 a computer method for predicting a ligand molecule's binding affinity and orientation to a
215 receptor protein. The active site prediction for target proteins (Keap1 and NF- κ B) gives the
216 grid box coordinates (x y z) of 17.500880 Å, 62.323000 Å, and 0.973748 Å; and -1.751769 Å, -
217 20.743853 Å, and -29.010438 Å, respectively (Fig. 3).

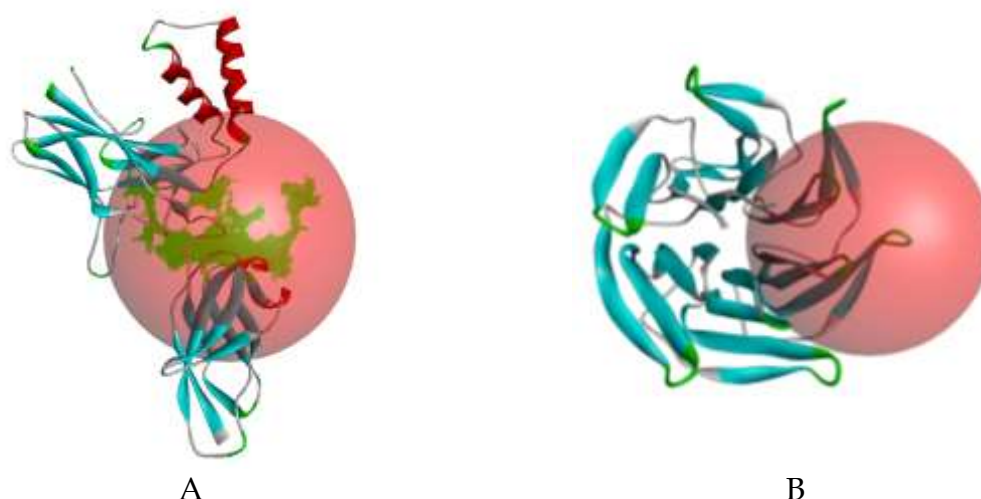


Fig. 3. Binding pocket (colored in red) generated using the BIOVIA Discovery Studio 2017 tool, coupled with the sequence containing the highlighted residues that constitute the binding pocket. A) NF- κ B (PDB ID 1A3Q) and B) Keap1 (PDB ID 4L7B).

218

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

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

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

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

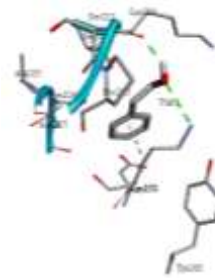
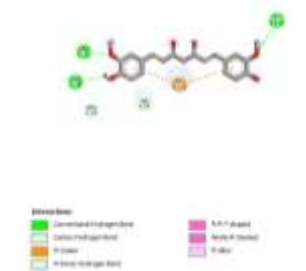
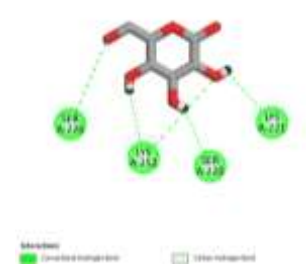
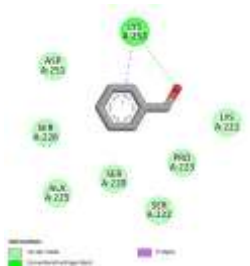
No.	Metabolites	PDB: 1A3Q Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μ M)	PDB: 4L7B Free Energy of Binding, ΔG (kcal/mol)	Inhibition Constant, Ki (μ M)
Volatile compounds					
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-4.19	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

240

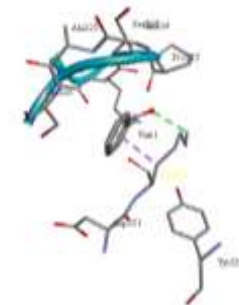
241 The theoretical binding modes of the top three metabolites with their target proteins (Keap1
 242 and NF- κ B) are shown in Figs. 4 and 5, respectively. The molecular docking results suggested
 243 that these metabolites interacted with the Keap1 and NF- κ B to form a complex through
 244 hydrogen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

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

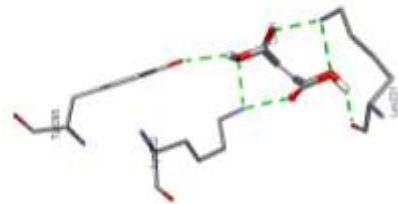
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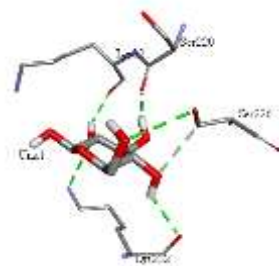
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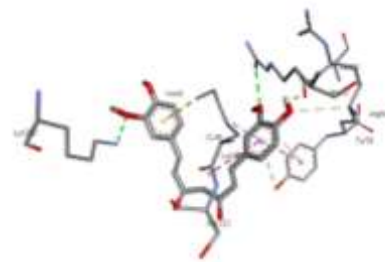
B



C



D



E

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

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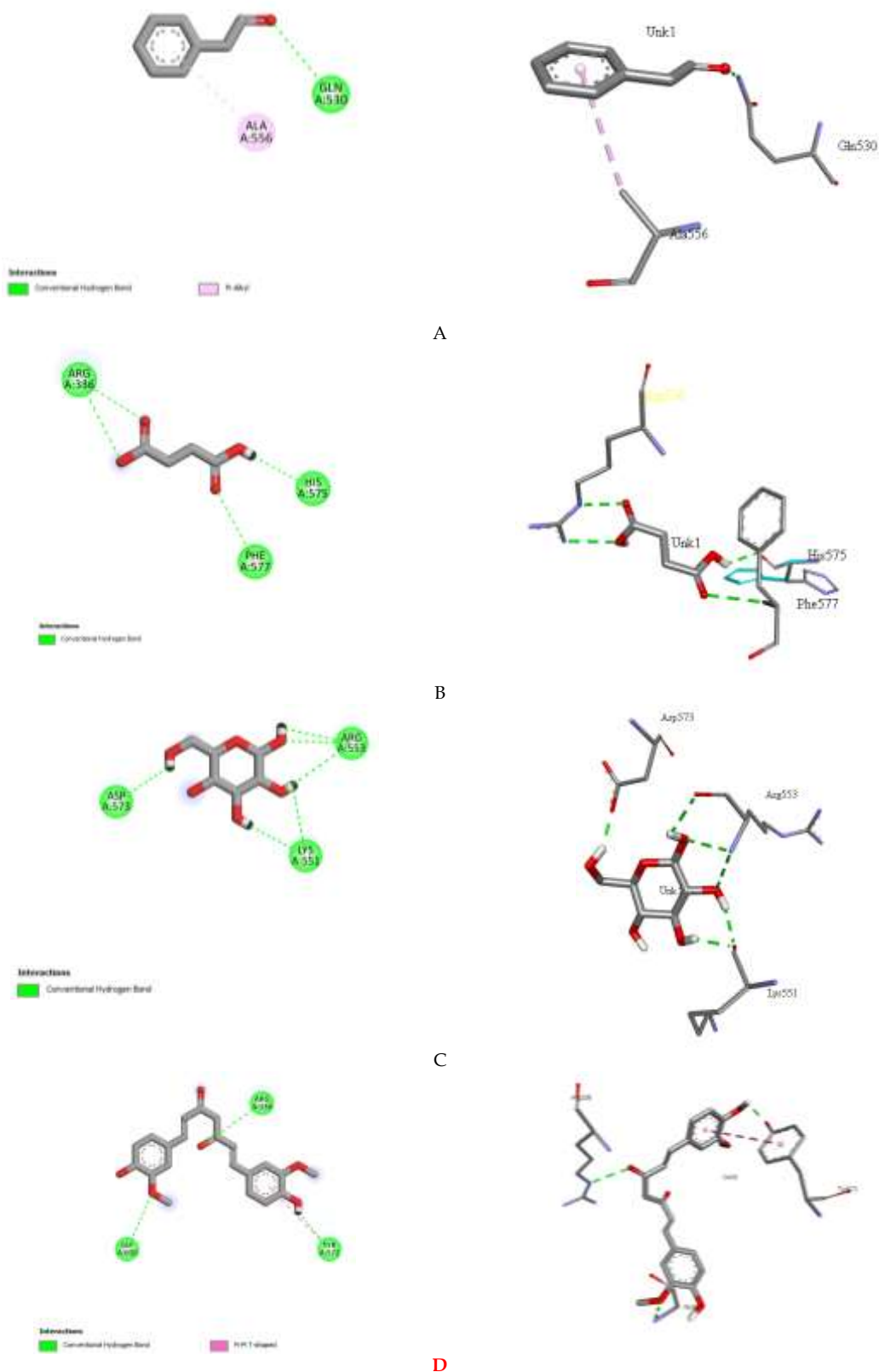


Fig. 5. 2D and 3D illustration of the type of interaction on the formation of Keap1's binding pocket (PDB ID 4L7B). A) 2-Phenylacetaldehyde, B) Succinic acid, C) Glucose, and D) Curcumin.

258

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

268 Curcumin (the reference compound) created three hydrogen bonds with the amino acid
269 residues ARG52, GLU58, and LYS252 to interact with the active site of NF- κ B. Meanwhile,
270 curcumin established three hydrogen bonds with the amino acid residues ARG336, TYR572,
271 and GLY600 to interact with the active site of nrf2 Keap1 (Figs. 4 and 5). Despite the fact that
272 curcumin could create three hydrogen bonds at both the active sites of NF- κ B and nrf2 Keap1,
273 its interaction with nrf2 Keap1 had a lower free energy of binding than phenylethanol,
274 benzaldehyde, and fumaric acid (Table 1). Overall, the metabolites of water kefir prefer to
275 interact with NF- κ B and nrf2 Keap1 receptors. Whereas fumaric acid and 2-
276 phenylacetaldehyde were metabolites that had the strongest interaction with NF- κ B (PDB ID
277 1A3Q) and nrf2 Keap1 (PDB ID 4L7B) receptors, respectively.

278

279 DISCUSSION

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

288 Carbon tetrachloride (CCl₄) is a component of the hepatotoxin, which acts after metabolic
289 activation and is extensively employed as a liver-damaging agent. In this study, the
290 administration of CCl₄ to animals caused hepatotoxicity, which was characterized by a
291 significant increase in ALT after several administrations. CCl₄ is metabolized by the enzyme
292 cytochrome p450 (CYP2E1) in the endoplasmic reticulum to a highly reactive trichloromethyl
293 radical. The trichloromethyl radical rapidly reacts with oxygen to form the trichloromethyl
294 peroxy radical, which rapidly reacts with lipids to form lipid peroxidation products. Free
295 radicals will disrupt the endoplasmic reticulum (ER) and lead to lipid accumulation, decreased
296 protein synthesis, and increased oxidase activity. CCl₄ hepatotoxicity is characterized by
297 hepatocellular necrosis with fat deposition (Ritesh et al., 2015). At the molecular level,
298 administration of CCl₄ can activate tumor necrosis factor (TNF)- α , nitric oxide (NO), and
299 transforming growth factor (TGF)- α and - β in cells, processes that precipitate cell self-
300 destruction or fibrosis. TNF- α leads to apoptosis, whereas TGF- β leads to fibrosis (Weber et
301 al., 2003).

302 In terms of its pathophysiological underpinnings, liver illness is linked to a condition known
303 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 (Elshagabee 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.

352 Molecular docking is a technique that is utilized in the context of NF- κ B and Nrf2 to make
353 predictions regarding the binding affinity and orientation of small-molecule inhibitors to their
354 active sites. The transcription factor known as NF- κ B is an essential component in the
355 management of both the immune system and the inflammatory response (Dai et al., 2021). The
356 expression of important inflammatory genes can be inhibited by small-molecule inhibitors that
357 impair the interaction between NF- κ B and DNA. These inhibitors have the potential to be used
358 in therapeutic applications. The Nrf2 binding site is Kelch-like ECH-associated protein 1 in the
359 context of nuclear factor erythroid 2-related factor 2, also known as Nrf2 (Keap1) (Zhao et al.,
360 2017; Jiang et al., 2019). Small-molecule inhibitors that disrupt the link between Keap1 and
361 Nrf2 can activate the Nrf2-ARE signaling pathway, which has been demonstrated to have
362 cytoprotective effects. Keap1 is a negative regulator of Nrf2, and this association can be
363 disrupted by small-molecule inhibitors (Zhao et al., 2017).

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

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

383 NF- κ B and Nrf2 are two transcription factors that play important roles in regulating
384 inflammation and cell survival. While NF- κ B is involved in the inflammatory response, and
385 Nrf2 is involved in the antioxidant response (Ganesh Yerra et al., 2013; Wang et al., 2022a).
386 Both transcription factors have been investigated as potential targets for the development of
387 hepatoprotective agents (Rahman et al., 2021; Gao et al., 2022; Wang et al., 2022a; Li et al.,
388 2023). There is evidence of crosstalk between the Nrf2 and NF- κ B pathways (Ganesh Yerra et
389 al., 2013; Gao et al., 2022). The Nrf2 pathway inhibits the activation of the NF- κ B pathway by
390 increasing antioxidant defenses and HO-1 expression, which efficiently neutralizes ROS and
391 detoxifies (Ganesh Yerra et al., 2013). The crosstalk between Nrf2 and NF- κ B could be a new
392 therapeutic target against hepatotoxicity (Gao et al., 2022). Researchers have tried to identify
393 molecule activators of Nrf2 as chemoprevention for ROS-dependent carcinogenesis, while
394 others have focused on identifying Nrf2 inhibitors to increase the sensitivity of cancer cells to
395 chemotherapy (Sharifi-Rad et al., 2023). While NF- κ B and Nrf2 are involved in different
396 cellular processes, they have both been investigated as potential targets for the development
397 of hepatoprotective agents. Molecular docking studies have been used to investigate the
398 interaction of potential hepatoprotective agents with these transcription factors. There is also

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

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

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

427 CONCLUSION

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

435

436 CONFLICT OF INTEREST

437 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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Hepatoprotective study of Indonesian water kefir against CCl₄-induced liver injury in rats

[Estudio hepatoprotector del kéfir de agua indonesio contra la lesión hepática inducida por CCl₄ 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 (CCl₄)-induced acute liver injury has not been studied.

Aims: 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: The present study aimed to investigate the hepatoprotective activity of water kefir in an animal model caused by 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 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-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 (CCl₄).

Objetivos: Evaluar la eficacia del kéfir de agua *in vivo* contra la lesión hepática aguda inducida por CCl₄ 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 CCl₄. 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 CCl₄. 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 (CCl₄)-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-

protective effects of water kefir in CCl₄-induced rats while also investigating the stability interactions of its metabolites within the NF- κ B 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 distilled 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 CCl₄ (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 ani-

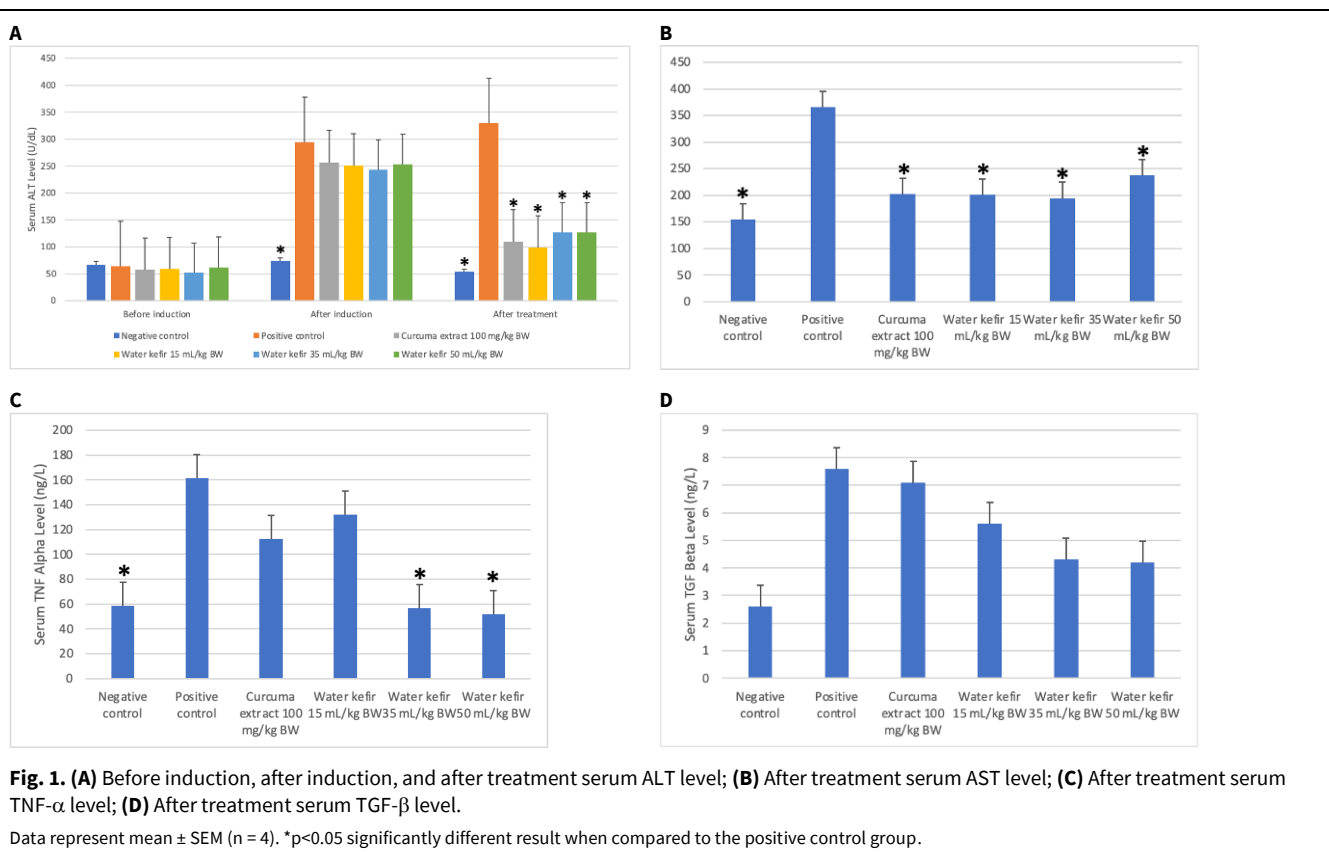
mals with liver injury, which is characterized by a significant increase in serum ALT level, as follows: (1) positive control group, (2) *Curcuma* extract group 100 mg/kg BW, (3) water kefir 15 mL/kg BW group, (4) water kefir 35 mL/kg BW group, and (5) water kefir 50 mL/kg BW group, with the addition (6) negative control group. Each group received group-specific treatment for two weeks, along with the administration of 1.25 mL/kg BW of CCl₄ (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- κ B (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.



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 $p < 0.05$.

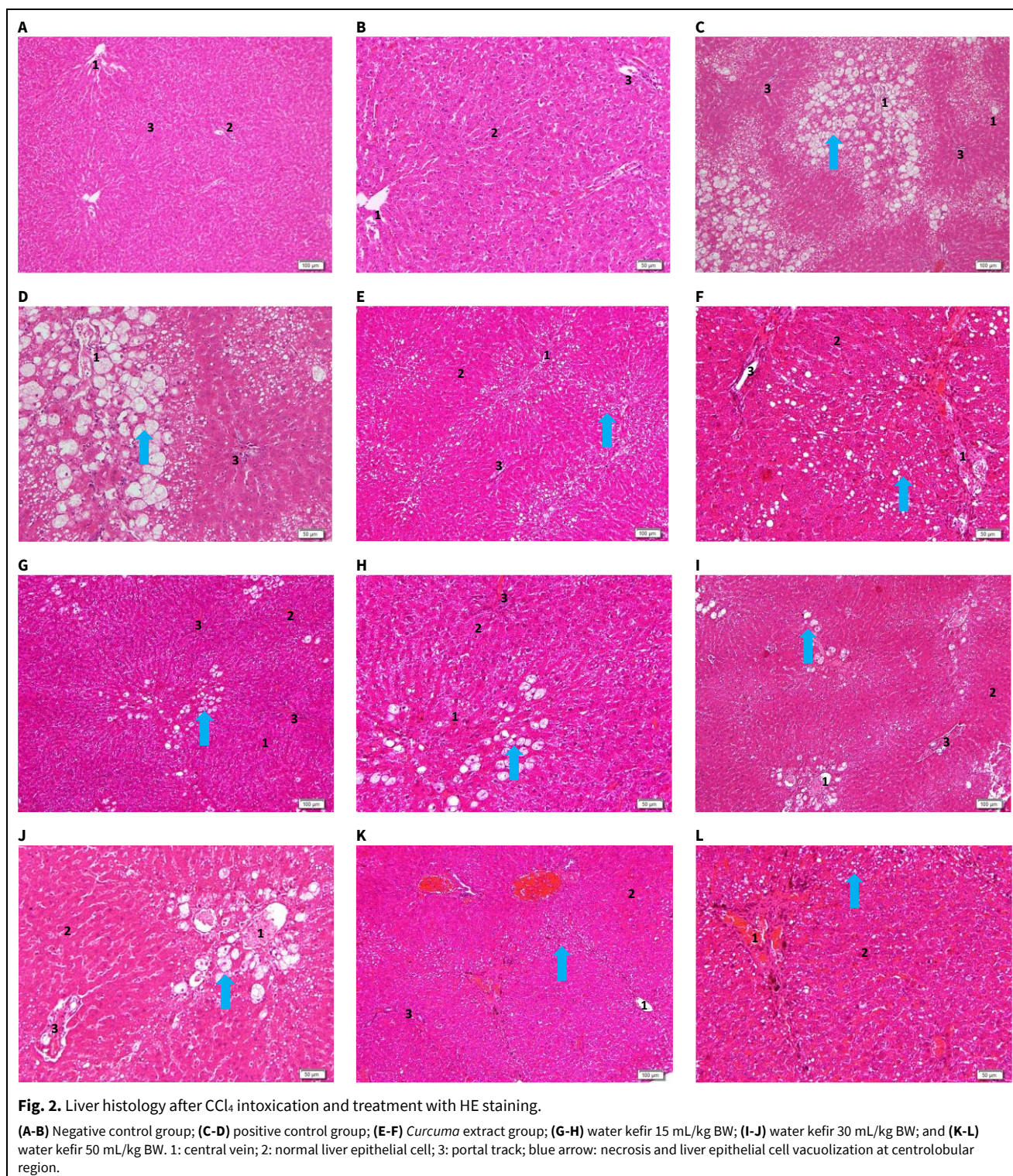
RESULTS

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 con-

trol 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

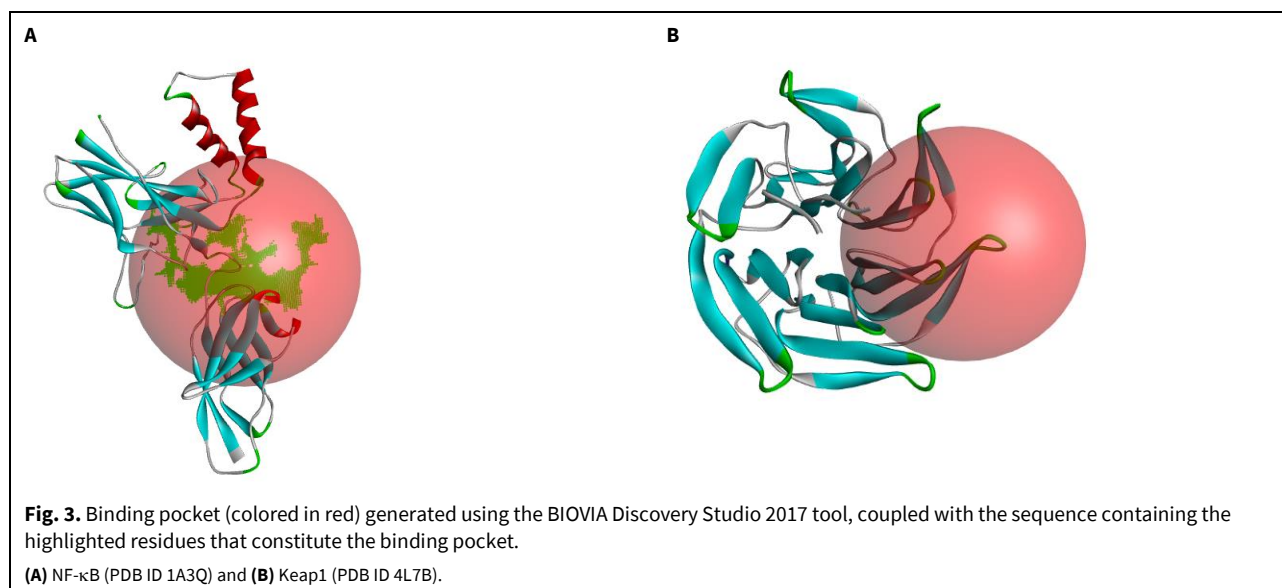


from level 1 (water kefir 50 mL/kg BW) (Fig. 2K-L) to level 2 [*Curcuma* extract (Fig. 2E-F), water kefir 15 mL/kg BW (Fig. 2G-H), and water kefir 30 mL/kg BW (Fig. 2I-J)].

Molecular docking

Molecular docking studies are considered a powerful tool for predicting the potential targets of bioac-

tive 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- κ B (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).

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).

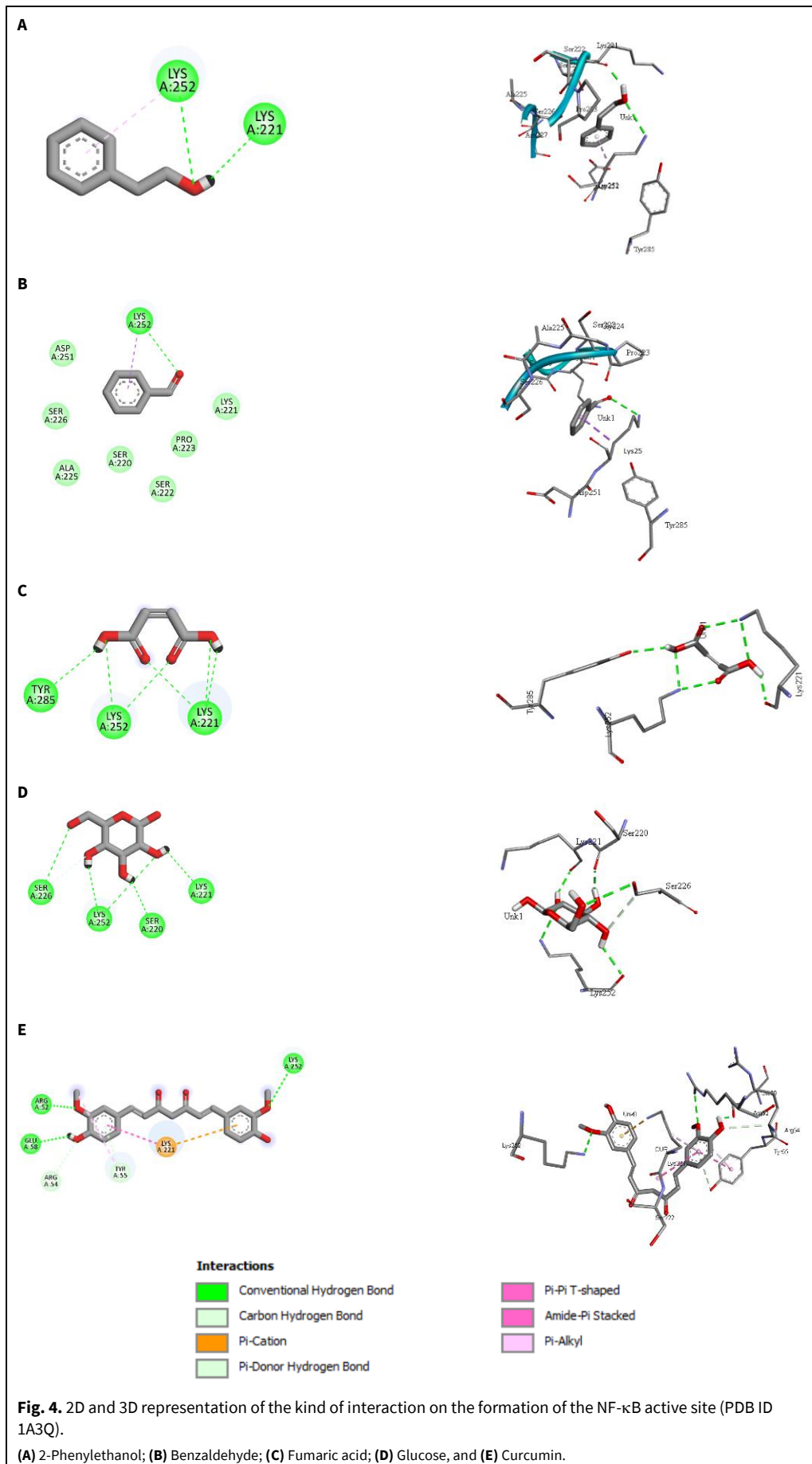
Table 1. The free energy of binding and inhibition constant of water kefir metabolite active site interaction on NF- κ B (PDB ID 1A3Q) and Keap1 (PDB ID 4L7B).

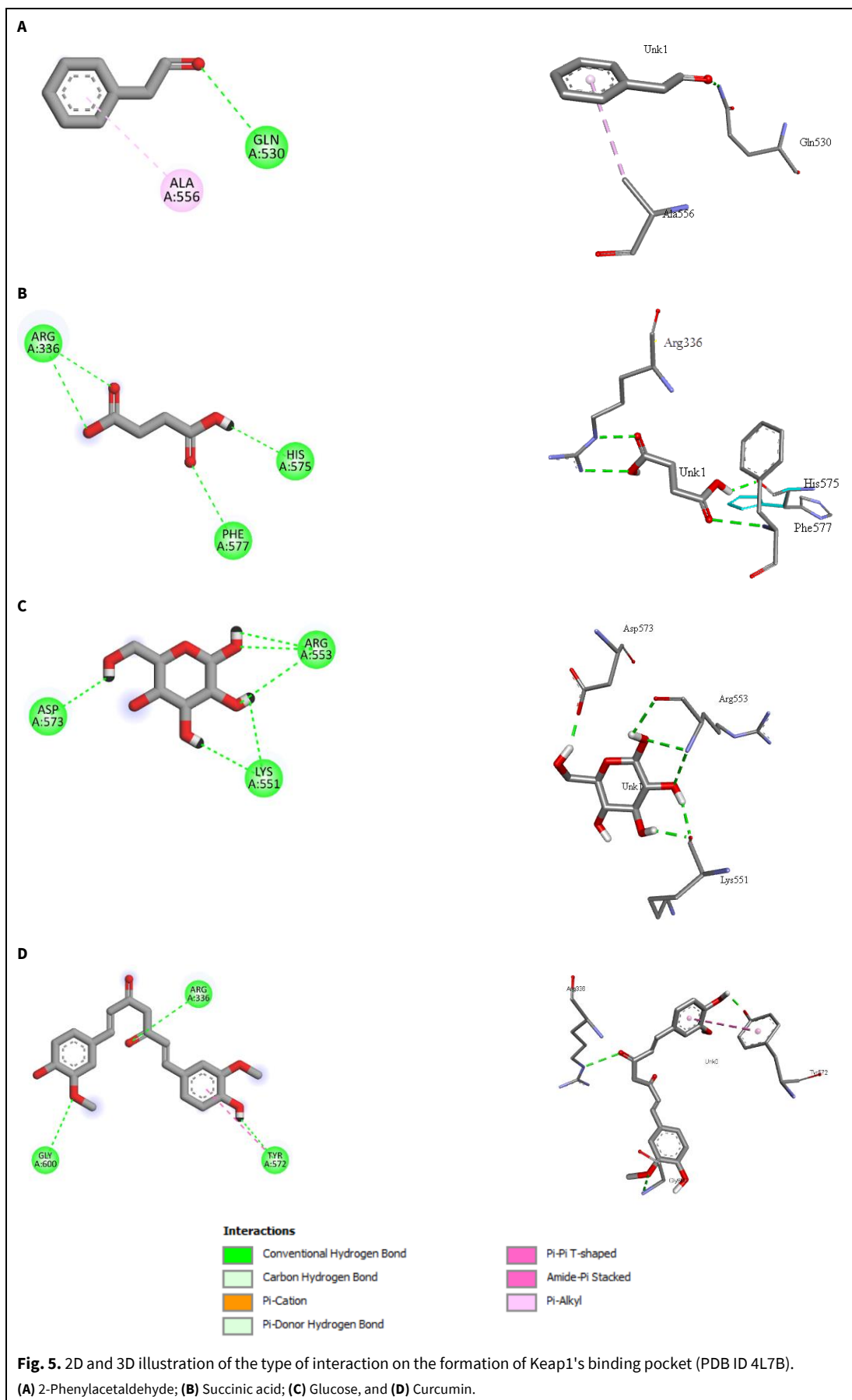
No.	Metabolites	PDB: 1A3Q		PDB: 4L7B	
		Free energy of binding, ΔG (kcal/mol)	Inhibition constant, K_i (μM)	Free energy of binding, ΔG (kcal/mol)	Inhibition constant, K_i (μM)
Volatile compounds					
1	2-Methylbutanol	-3.31	3,770	-2.68	10,890
2	3-Methylbutanol	-3.22	4,360	-2.50	14,670
3	2-Phenylethanol	-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,





GLU58, and LYS252 to interact with the active site of NF- κ B. 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- κ B 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- κ B and nrf2 Keap1 receptors. Whereas fumaric acid and 2-phenylacetaldehyde were metabolites that had the strongest interaction with NF- κ B (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 (CCl₄) 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 CCl₄ to animals caused hepatotoxicity, which was characterized by a significant increase in ALT after several administrations. 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 peroxy 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 (Ritesh et al., 2015). 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 (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 (El-shaghabee 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- κ B 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- κ B 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- κ B 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 K_i are important parameters used in molecular docking to evaluate the strength of the interaction between a ligand and a receptor pro-

tein. 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). K_i , 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 K_i are used to predict the binding affinity and selectivity of a ligand to a receptor protein (Du et al., 2016). K_i , 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- κ B (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- κ B and Nrf2 are two transcription factors that play important roles in regulating inflammation and cell survival. While NF- κ B 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- κ B pathways (Ganesh Yerra et al., 2013; Gao et al., 2022). The Nrf2 pathway inhibits the activation of the NF- κ B 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- κ B 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- κ B 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- κ B pathways, which could be a new therapeutic target against hepatotoxicity.

In the pathogenesis of hepatocellular damage, liver fibrosis, and HCC, NF- κ B 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- κ B 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- κ 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- κ B-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 unrestrictedly. When HSCs have been activated, NF- κ B 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- κ B 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 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- κ B 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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