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3 **Extracts of bilberry (*Vaccinium myrtillus* L.) fruits improve liver steatosis and injury in**
4 **mice by preventing lipid accumulation and cell death**

5

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38

39 **KEYWORDS**

40 Bilberry fruits; fatty liver; liver injury; non-alcoholic steatohepatitis (NASH); *Vaccinium*

41 *myrtillus*

42

43

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45 **ABSTRACT**

46 Bilberry has been reported to have anti-oxidant and anti-inflammatory properties. We studied
47 the effect of bilberry (*Vaccinium myrtillus* L.) fruits extracts (BEs) on the pathogenesis caused
48 by lipid accumulation in fatty liver and non-alcoholic steatohepatitis (NASH). 5 µg/ml of BEs
49 was enough to suppress lipid accumulation in the fatty liver model of the mouse hepatic
50 AML12 cells. BEs increased cell viability and anti-oxidant capacity, presumably by activating
51 (phosphorylating) Akt/STAT3 and inducing MnSOD/catalase. BEs also significantly reduced
52 Rubicon and induced p62/SQSTM1, possibly contributing to reduce cellular lipids
53 (lipophagy). When the mice were fed supplemented with BEs (5% or 10%, w/w), hepatic
54 steatosis, injury, and hypercholesterolemia/hyperglycemia were significantly improved.
55 Furthermore, histological and cytokine studies indicated that BEs possibly suppress hepatic
56 inflammation (hepatitis) and fibrosis. Therefore, BEs improved liver steatosis and injury, and
57 potentially suppress fibrosis by suppressing inflammatory response, which therefore may
58 prevent the progression of fatty liver to NASH.

59 The perennial dwarf shrub bilberry (*Vaccinium myrtillus* L.) is also known as the European
60 blueberry and is widely grown in the northern hemisphere across Europe and Central Asia [1].
61 Although bilberry leaves are a promising source of bioactive natural products [2], bilberry
62 fruits are better known for the health benefits of its phytochemicals. Bilberry fruits are rich in
63 anthocyanidins and anthocyanins [3] and other potent natural antioxidants like flavanols and
64 phenolic compounds. Several studies have shown that *V. myrtillus* berries contain higher
65 anthocyanin and antioxidant levels than cultivated blueberries (*V. corymbosum*) [4].

66 Wild bilberry fruits are well established in pharmacognosy and have been used as a
67 food source for centuries. Bilberry is a rich source of anthocyanins [3] and its extracts are
68 extensively used in dietary supplements and pharmaceutical products. Bilberry fruits extracts
69 are antioxidant [5-8], can decrease capillary permeability and fragility [9], inhibit platelet
70 aggregation [10], and strengthen collagen matrix cross-linkages [11,12]. Bilberry crude
71 extracts are used in the treatment of ophthalmological diseases and blood vessel disorders
72 [13]. Recently, they have been administered to treat dysentery, diarrhea, and mouth and throat
73 inflammations [14]. Certain studies have shown that the bilberry anthocyanins may prevent
74 metabolic syndrome [15-22]. However, the mechanisms by which bilberry influences diabetes
75 and lifestyle-related diseases have not yet been identified. Since bilberry seems to prevent
76 diabetes mellitus, it may also prevent non-alcoholic steatohepatitis (NASH), which is a
77 progressive and severe form of non-alcoholic fatty liver disease (NAFLD). Fat accumulation
78 in liver is involved in the onset and progression of these conditions [23,24].

79 In the present study, we investigated the effects of dried bilberry (*Vaccinium*
80 *myrtillus*) fruits extracts (BEs) especially on mouse hepatic steatosis and injury in cellular and
81 animal models. We elucidated the possible mechanisms of BEs on the prevention of fatty liver
82 and NASH, with regards to hepatic steatosis and injury. Also, we proposed the possible
83 molecular mechanisms of BEs effects on the prevention of fatty liver and NASH, regarding
84 autophagy, fat metabolism, oxidative stress and mitogenesis.

85

86 **Materials and methods**

87 *Cell culture, reagents and bilberry fruits extracts (BEs)*

88 Alpha mouse liver 12 cells (AML12; ATCC[®] CRL-254[™], Manassas, VA, USA) were
89 maintained at 37 °C and 5.0% CO₂ in DMEM/Nutrient Mixture F-12 (Nacalai Tesque, Kyoto,
90 Japan) supplemented with 10% fetal bovine serum (FBS) and 1× ITS-A
91 (Insulin-Transferrin-Selenium-Sodium Pyruvate Supplement, Thermo Fisher Scientific,
92 Waltham, MA, USA). Fatty acids (FA; oleic and linoleic) and T0901317 (T090; LXR α
93 agonist) were purchased from TCI Chemicals (Tokyo, Japan) and Sigma-Aldrich (St. Louis,
94 MO, USA), respectively. To induce steatosis of hepatocytes, T090 (1 μ M) or FA (100 μ M for
95 each oleic and linoleic acids) were added to the culture media daily for 3 or 5 days [25-27].

96 A 90% ethanolic extracts of bilberry (*Vaccinium myrtillus* L.) fruits was purified with
97 an adsorbent resin to obtain bilberry fruits extracts containing \geq 36% anthocyanin glycosides.
98 The purified product was lyophilized, homogenized, and used as bilberry fruits extracts (BEs).
99 BEs were added daily to the culture media at the designated concentration. We used BEs in
100 the concentrations from 1 μ g/ml to 10 μ g/ml, which was proved to show no cytotoxicity to
101 AML12 hepatocytes by lactose dehydrogenase (LDH) assay using LDH Cytotoxicity
102 Detection Kit (Takara Bio Inc., Shiga, Japan) (Figure S1).

103

104 *Hepatic lipid accumulation analysis*

105 AML12 cells were rinsed with PBS, fixed with 4% paraformaldehyde/PBS for 5 min, and
106 stained with Nile red (AdipoRed Assay Reagent, Lonza, Basel, Switzerland). After incubation
107 at room temperature for 10-15 min, cells were quantified and observed for fluorescence with
108 excitation at 485 nm and emission at 572 nm (expressed as relative fluorescence units, RFU)
109 in a multimode plate reader (Infinite[®] 200 PRO, TECAN, Zurich, Switzerland) and under a

110 fluorescence microscope (Biozero; Keyence, Osaka, Japan). Hoechst33342 (Sigma-Aldrich)
111 was used for nuclear counterstaining. Relative lipid accumulation (RLA) was determined as
112 the ratio of AdipoRed to Hoechst33342 intensity values to calculate well-level normalization.
113 For all doses, the average RLAs of the technical replicates were calculated and normalized to
114 vehicle control wells to determine the mean fold-change values across the test plates. The
115 mean dose-response and standard error were derived from eight independent experiments.

116 For animal experiments, liver triacylglycerol (TG) contents were measured with an
117 adipogenesis colorimetric/fluorometric assay kit (BioVision, Milpitas, CA, USA) according to
118 the manufacturer's instructions [28]. The TG concentration was calculated from a standard
119 curve and normalized to mass per μg protein.

120

121 *Monitoring and evaluation of hepatocyte growth (survival and proliferation)*

122 The effects of BEs on the growth (survival/proliferation) of non-steatotic or steatotic AML12
123 cells were determined with the xCELLigence System (Roche, Basel, Switzerland). The
124 survival/proliferation analyses of non-steatotic and steatotic hepatocytes were performed in
125 the following way. Briefly, for the non-steatotic hepatocytes, untreated AML12 cells were
126 plated at a density of 1.5×10^4 cells/well in an E-Plate 16 PET (ACEA Biosciences, San
127 Diego, CA, USA), and for the steatotic hepatocytes, pre-steatotic AML12 cells were plated at
128 a density of 1.2×10^3 cells/well in the plate. The plates were then placed in the xCELLigence
129 System and BEs were administered daily at the designated concentrations. The
130 above-mentioned experiments were monitored by real-time quantitation.

131

132 *Evaluation of cellular redox states in vitro*

133 Cellular redox states were imaged/evaluated with fluorescence microscopy using
134 Redox-sensitive roGFP as previously described [29].

135

136 *RNA extraction and qRT-PCR*

137 Total hepatocyte RNA was isolated with an Aurum Total RNA Fatty and Fibrous Tissue Kit
138 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cDNA was synthesized with an iScript
139 cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the
140 manufacturer's instructions. The quantitative real-time PCR (qRT-PCR) was performed on the
141 Bio-Rad CFX Connect System with SsoAdvanced Universal SYBR Green Supermix
142 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The used primers were listed in Table 1.
143 The qRT-PCR data were analyzed by the comparative Ct Method ($2^{-\Delta\Delta C_t}$) [30]. The mRNA
144 expressions were normalized to *36B4* (acidic ribosomal phosphoprotein P0 (RPLP0)) mRNA
145 and the relative mRNA expression levels were calculated using the control mRNA.

146

147 *Animal care, experimental design, and sample preparation*

148 Male homozygous leptin receptor-deficient (BKS.Cg-+ Leprdb/+ Leprdb/Jcl; db/db) mice (10
149 weeks) were obtained from CLEA Japan (Tokyo, Japan). The mice were divided into four
150 groups. Group 1 received a normal control diet. Group 2 received a high-fat and
151 high-cholesterol (HF&HC) diet. Groups 3 and 4 received HF&HC diet supplemented with 5%
152 and 10% BEs, respectively. The HF&HC diet (No. D09100301) was purchased from Research
153 Diet Inc. (New Brunswick, NJ, USA), mixed with BEs, and freshly prepared every 2 days.

154 The mice were sacrificed before BEs administration and after 8 weeks to collect liver
155 and blood specimens. The liver/body weight ratios were calculated to estimate relative
156 changes in liver mass. Liver specimens were prepared for histological and western blot
157 analyses. Blood specimens were used in biochemical analyses (alanine
158 transaminase/glutamate pyruvate transaminase (ALT); aspartate transaminase/glutamate
159 oxaloacetate transaminase (AST); glucose (GLU); and total cholesterol (T-CHO). The animal
160 care and experimental procedures were approved by the Animal Research Committee of
161 Hokkaido University (Permission No. 16-0120) and performed in strict accordance with the

162 Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. All efforts
163 were made to minimize animal suffering.

164 For histological analysis, liver specimens were fixed in 10% buffered formalin,
165 embedded in paraffin, and stained with hematoxylin and eosin (H&E) or Azan. To visualize
166 hepatic lipid accumulation, frozen sections of formalin-fixed liver tissue were stained with
167 Sudan III. Briefly, frozen liver sections (8 μ m thick) were mounted on slide glasses, air-dried,
168 and rinsed with 50% v/v ethanol. Next, the specimens were stained in Sudan III stain for 10
169 min at room temperature and rinsed with 50% (v/v) ethanol to remove excess stain.
170 Hematoxylin nuclear counterstain was applied for 3 min. The stained liver sections were then
171 washed several times in water, mounted with a coverslip, and viewed under a microscope.

172

173 *Immunoassay*

174 For expression analysis of proteins associated with autophagy (lipophagy) and fatty acid (FA)
175 synthesis, antibodies against the following proteins were used; Rubicon, p62/SQSTM1 (Cell
176 Signaling Technology, Danvers, MA, USA), FASN (fatty acid synthase, BD Transduction
177 Laboratories, Franklin Lakes, NJ, USA) and GAPDH (Cell Signaling Technology, Danvers,
178 MA, USA) using standard western blot analysis protocol.

179 For expression analysis of proteins associated with autophagy, survival/proliferation,
180 and antioxidant properties, protein separation and detection were performed using an
181 automated capillary electrophoresis system, Wes™ (ProteinSimple, San Jose, CA, USA) and
182 antibodies against the following proteins were used; p62/SQSTM1, phospho-Akt, Akt,
183 phospho-STAT3 (Tyr705) (Cell Signaling Technology, Danvers, MA, USA), STAT3 (Santa
184 Cruz Biotechnology, Dallas, TX, USA), manganese-dependent superoxide dismutase
185 (MnSOD) (BD Transduction Laboratories, Franklin Lakes, NJ, USA), catalase (EMD
186 Biosciences, Darmstadt, Germany), and GAPDH. Signals were detected with an
187 HRP-conjugated secondary anti-rabbit or anti-mouse antibody, and were visualized using

188 Compass for ProteinSimple software.

189

190 *Statistical analysis*

191 All results were expressed as means \pm standard error of the mean (SEM). ANOVA followed
192 by Tukey–Kramer test were performed to compare the means. Statistical difference at $p <$
193 0.05 were considered as significant.

194

195 **Results**

196 *Effects of BEs on hepatic cells*

197 To determine the effects of BEs on hepatocyte steatosis, we created a steatotic murine
198 hepatocytes in 2 ways by adding fatty acids (FA; oleic and linoleic) or a nuclear receptor
199 LXR α agonist (T090) to AML12 cells. Figure 1A shows lipid accumulation induced by FA or
200 T090, which are standardized by untreated AML12 cells (left panel). Fluorescence
201 photomicrographs of cells showing lipids (green color) and nuclei (red color) are shown in
202 Figure 1A (right panel). Both FA and T090 induced lipid accumulation in AML12 cells,
203 though FA was more efficient. Cell growth (survival/proliferation) of the FA- and
204 T090-induced steatotic AML12 cells was significantly lower than that of the untreated
205 controls (Figure 1B). The addition of FA and T090 both inhibited the proliferation of AML12
206 cells, but the inhibitory effect of FA was observed at an early stage of proliferation, whereas
207 that of T090 was observed at a later stage of proliferation. However, steatosis was
208 significantly improved by adding BEs into the culture media. This effect of BEs was most
209 apparent at 5 $\mu\text{g/mL}$ BEs, indicating an optimum concentration for this effect (Figure 1C).
210 BEs seem to suppress cellular lipid accumulation by both types of hepatic steatosis (FA and
211 T090) dose-dependently (Figure 2A).

212 We examined the pro-survival effects of BEs in non-steatotic AML12 cells. BEs
213 significantly promoted cell survival/proliferation showing a peak at 1 $\mu\text{g/mL}$ (Figure 2B).

214 Oxidative stress (H₂O₂, 500 μM) reduced cell survival in non-steatotic AML12 cells, which
215 was improved significantly by the treatment with BEs (Figure 2C). These results indicate that
216 BEs confer a pro-survival property and a resistance against oxidative stress to hepatocytes.
217 Therefore, we monitored changes of reactive oxygen species (ROS) levels over time in
218 AML12 cells treated with H₂O₂. Interestingly, there were no significant differences between
219 cells added with and without BEs in terms of their cellular ROS levels (Figure 2D). These
220 data may indicate that BEs do not directly eliminate intracellular ROS, but BEs provide the
221 resistance against oxidative stress. Although Nrf2, activated by oxidative stress and toxic
222 substances in the environment, is well known to protect cells from environmental stresses
223 including ROS, significant activation of an antioxidative Nrf2-DNA binding was not observed
224 (data not shown).

225

226 *Effect of BEs on mouse with fatty liver*

227 Next, we challenged BEs against mouse fatty liver model to confirm the BEs' *in vivo* effects.
228 HF & HC diet markedly increased liver/body weight ratios, hepatic steatosis/TG contents, and
229 injuries, which were suppressed by BEs in a dose-dependent manner (Figures 3A, 3B, 3D).
230 There was no significant difference in dietary intake between the groups receiving BEs and
231 those not receiving them (Table 2). Histological study and hepatic TG measurement revealed
232 that BEs administration undoubtedly suppressed fat accumulation/TG contents in mouse liver
233 (Figure 3B). The HF & HC diet caused mild fibrosis around portal area in mouse livers
234 (Figure 3C, arrowhead), whereas those being administered BEs presented much less fibrosis.
235 Blood biochemistry of the mice fed with the normal and HF & HC diet are shown in Figure
236 3D. Plasma ALT and AST levels in the 5% BEs group were significantly lower than those in
237 the group receiving HF & HC diet alone ($p < 0.05$). Plasma GLU and T-CHO levels for the
238 10% BEs group were also significantly lower than those for the HF & HC diet group.
239 Furthermore, compared with those given the normal diet, a series of pro-inflammatory

240 cytokine levels (e.g. TNF- α , IL-9, IL-1 β and IFN- γ) were higher in mice receiving the HF &
241 HC diet, which were likely to be reduced by BEs administration though no significant
242 difference was found among them (Figure S2).

243 These data indicate that BEs improve hyperglycemia/hypercholesterolemia, liver
244 injury and inflammation, and presumably liver fibrosis.

245

246 *Effects of BEs on the expression levels of the genes and proteins regulating lipid metabolism,*
247 *survival/proliferation, antioxidant, and autophagy in hepatocytes*

248 The mouse fatty liver model clearly showed that BEs improve fat accumulation, injury,
249 inflammatory reaction, and fibrosis of liver. To elucidate the underlying molecular
250 mechanisms, we examined the expressions of the genes and proteins related to these events
251 using mouse AML12 cells.

252 Figure 4A shows the protein expressions of autophagy- and fat
253 metabolism-associated proteins including Rubicon (run domain beclin-1-interacting and
254 cysteine-rich domain-containing protein, a cellular protein that suppresses late stage of
255 autophagy), p62/SQSTM1 (an autophagy marker), and FASN (fatty acid synthase) [31-34] in
256 AML12 cells. Fat accumulation in hepatocytes was stimulated by the addition of FA and T090
257 to the culture media (Figure 1A). When AML12 cells were subjected to FA, Rubicon
258 expression was dramatically elevated. The addition of BEs downregulated Rubicon
259 significantly and concurrently upregulated p62/SQSTM1 expression significantly (Figure 4A,
260 upper panel). Therefore, it is suggested that BEs enhanced autophagy (lipophagy) by
261 suppressing Rubicon expression, and eventually reduced lipid accumulation. T090 clearly
262 upregulated FASN, which was certainly suppressed by the addition of BEs. On the contrary,
263 FASN was neither induced in FA-treated AML12 cells, nor affected by BEs administration at
264 all (Figure 4A, upper panel). Taken together, BEs may improve fat accumulation by

265 enhancing autophagy (lipophagy) or suppressing FA synthesis in the steatotic hepatocytes,
266 depending on the causes of steatosis.

267 We then studied the expression of genes related to FA synthesis including *LXR* (a
268 member of the family of nuclear receptor transcription factors involved in lipid synthesis),
269 *SREBP-1c* (transcription factor suppressed in FA, TG, and cholesterol synthesis), *FASN*, and
270 *ACC-1* (acetyl-CoA carboxylase, a key enzyme for FA synthesis). As shown in Figure 4B,
271 these four genes were upregulated by LXR stimulus (T090), which were significantly
272 suppressed by BEs administration except *LXR*. Therefore, BEs suppress cellular FA synthetic
273 pathway stimulated by LXR agonist. In case where the cells were stimulated by FA, BEs did
274 not affect the expression levels of *LXR*, *SREBP-1c*, *FASN*, or *ACC-1* (data not shown).

275 To elucidate the molecular mechanisms of BEs for autophagy, survival/proliferation,
276 and antioxidant properties, we additionally examined the following protein expressions in
277 non-steatotic AML12 cells (Figure 4C). p62/SQSTM1 protein was increased very mildly and
278 transiently after the BEs treatment even in non-steatotic cells (day 3). This suggests that BEs
279 did not directly affect p62/SQSTM1 expression in non-steatotic cells not expressing Rubicon
280 (Figure 4C), and the increase of p62/SQSTM1 (Figure 4A) was secondary to reduction of
281 Rubicon. Akt and STAT3 were both phosphorylated after the BEs treatment with slight
282 increase of these protein expressions. Regarding the antioxidant proteins, the increase of
283 protein expression was observed in MnSOD and catalase (Figure 4C).

284

285 **Discussion**

286 In the present study, we demonstrated that BEs improve fatty liver and the following liver
287 injury using mouse liver cells and fatty liver model. We proposed the unique mechanisms of
288 BEs' these effects on autophagy (Rubicon and p62/SQSTM1), lipid metabolism (*FASN*,
289 *STAT3*), survival/proliferation (*Akt/STAT3*), and antioxidant (*MnSOD/catalase*).

290 We showed that BEs reduced the oxidative injury induced by hydrogen peroxide

291 (Figure 2C). BEs did not eliminate ROS directly (Figure 2D), but BEs induced the antioxidant
292 proteins, MnSOD and catalase significantly (Figure 4C). Although Nrf2 is known to
293 eventually confer resistance against oxidative stress to cells, significant activation of Nrf2 was
294 not observed (data not shown). Though anthocyanin rich in BEs is known to suppress ROS
295 production and prevent resulting toxic injury [35], it is not clear whether anthocyanin plays a
296 central role for BEs' these effects.

297 We also showed that BEs may affect hepatocyte survival and/or proliferation. BEs
298 improved cell viability in both non-steatotic and steatotic cells (Figures 1C, 2B, & 2C). As
299 shown in Figure 4C, Akt and STAT3 seem to be both phosphorylated after the BEs treatment
300 with slight increase of these protein expressions. The activation of these proteins by BEs
301 should contribute to cell survival and proliferation in steatotic and non-steatotic cells. These
302 data indicate that BEs may have pro-survival and regenerative potentials of tissues/organ in
303 various conditions, though further investigation is required.

304 As we have already shown that STAT3 activation suppressed steatosis by inhibiting
305 lipogenic genes such as SREBP-1c [36], a STAT3/SREBP pathway would definitely play an
306 important role for the suppression of hepatic steatosis. Therefore, an activation of STAT3 may
307 contribute at least partly to reduction of lipid accumulation by BEs.

308 We carefully examined the influence of BEs on fatty liver using both cultured liver
309 cells and mouse liver. BEs suppressed lipid accumulation and its progression in both models
310 (Figures 2A, 3B). BEs suppressed lipid accumulation in two different models (FA addition
311 and TG synthesis) (Figures 4A, 4B) and enhanced lipolysis by autophagy (FA addition model)
312 (Figure 4A). Most notable was Rubicon, which suppresses the late stage of autophagy and
313 accelerates lipid accumulation [34]. It suppresses autophagosome fusion with lysosomes,
314 which is the final step of autophagy. The rate of autophagy decreases with increasing
315 intracellular Rubicon levels. Therefore, fatty liver may have progressed with increased hepatic
316 Rubicon levels suppressing lipophagy. In the present study, we first report that BEs markedly

317 downregulate Rubicon and promote lipophagy (shown by an increase of p62/SQSTM1) in
318 FA-induced steatotic hepatocytes [32,33]. BEs also reduced FASN expression, which is
319 directly related to fat accumulation [31]. Therefore, BEs are predicted to suppress lipid
320 accumulation in the liver by promoting lipolysis (decrease of Rubicon) and inhibiting TG
321 synthesis (decrease of FASN). Because p62/SQSTM1 is also known to possess a
322 cytoprotective effect [28], the increase of p62/SQSTM1 by BEs may also contribute to
323 prevention against liver injury.

324 It has been reported that bilberry is antioxidant [5-8] and anti-inflammatory [37-39].
325 Also, in the present study, BEs showed antioxidant and anti-inflammatory properties other
326 than defatting and protective property. This may suppress the steatosis-induced hepatic
327 inflammation (hepatitis). As shown in Figure S2, BEs restrained biological effects against
328 inflammatory cytokines such as TNF- α , IL-9, IL-1 β and IFN- γ . This means that BEs have
329 inhibitory effects against inflammation as well as defatting and protection, suggesting that
330 BEs potentially prevent the progression from fatty liver to NASH. It is interesting to know
331 that BEs may control these chemokine activities directly or indirectly, which may be
332 important clinical targets of the future study of BEs.

333 Morrison *et al.* [40] comprehensively investigated the effects of Mirtoselect, an
334 anthocyanin-rich bilberry extracts, on NASH and associated fibrosis. They examined the
335 effects of anthocyanins-rich bilberry extracts on late pathology of NASH by animal
336 experiment only. They concluded that Mirtoselect reduced development of NASH, attenuating
337 both steatosis and inflammation as well as the development of hepatic fibrosis. On the other
338 hand, we focused on the effect of BEs on earlier pathological events of fatty liver developing
339 into NASH. We also studied its mechanism of action in detail by cell experiments, focusing
340 on cell survival/proliferation, fat accumulation (incorporation, degradation), antioxidant, and
341 inflammation. The results obtained from cell experiments were verified by animal
342 experiments. Our study gives new mechanistic/therapeutic insights on the effect of bilberry

343 for the liver pathology developing into NASH.

344

345 **Conclusion**

346 Overall, we identified the factors that improve lipid accumulation, cytotoxicity, oxidative
347 stress, and inflammation by BEs. Because hepatocyte fat accumulation, insulin resistance,
348 oxidative stress, injury and sugar/fat metabolism are of particular importance in NASH
349 progression, BEs was proved to target some of these crucial factors. We also showed that BEs
350 potentially suppresses inflammation and fibrosis induced by fat accumulation and injury of
351 liver. Based on the results detailed above, bilberry is expected to be a liver-friendly food
352 supplement.

353

354 **Author Contribution**

355 M. O. designed and supervised the study. N. M., S. H. and M. O. wrote and revised the
356 manuscript. S. H., Y. S. J. and H. Y. performed cell experiments. S. H. and M. O. performed
357 animal experiments. S. H., T. S., N. M. and M. O. analyzed and discussed the results. All
358 authors gave final approval and consented to be accountable on all matters.

359

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362

363 **Disclosure statement**

364 The authors declare no conflict of interest.

365

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373

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479 **Table 1.** Primer list for the quantitative real-time PCR (qRT-PCR).

Symbol	Gene name	Primer sequence
LXR	Liver X receptor α	F: 5'-CTGGGGTTGCTTTAGGGATAG-3' R: 5'-TTCCGCTTTTGTGGACGAAG-3'
SREBP-1c	Sterol regulatory element-binding protein-1c	F: 5'-AAGCTCAAGCAGGAGAACC-3' R: 5'-ATGCCCTCCATAGACACATC-3'
FASN	Fatty acid synthase	F: 5'-GGGCTCTATGGATTACCC-3' R: 5'-AAAAGGAGGCGTCGAAC-3'
ACC-1	Acetyl-CoA carboxylase	F: 5'-GGCTGCTAAGGTGGAAGTAGG-3' R: 5'-TGCCTGATGATCGCACGAAC-3''
36B4	Acidic ribosomal phosphoprotein P0	F: 5'-ATGGGTACAAGCGCGTCCTG-3'' R: 5'-GCCTTGACCTTTTCAGTAAG-3'

480 F, forward; R, reverse

481 **Table 2.** Body weight and food intake in mice fed a high-fat and high-cholesterol (HF&HC)
482 diet with/without bilberry fruits extracts (BEs) for 8 weeks. Data are means \pm SEM; n = 4-8
483 per group. * $p < 0.05$ vs. HF&HC group. There were no significant differences in initial body
484 weight or food intake between groups.

	HF&HC	5% BEs + HF&HC	10% BEs + HF&HC
Initial body weight (g)	37.58 \pm 0.80	38.60 \pm 0.74	39.15 \pm 0.48
Final body weight (g)	49.83 \pm 1.01	46.37 \pm 1.60	44.43 \pm 1.17*
Food intake (g/d)	3.89 \pm 0.13	3.49 \pm 0.10	3.21 \pm 0.10

485

486 **Figure legends**

487

488 **Figure 1.** Bilberry fruits extracts (BEs) improved steatotic hepatocyte growth
489 (survival/proliferation) (AML12 cells). (A) Administration of free fatty acids (FA) consisting
490 of 100 μM oleic and linoleic acids or 1 μM T0901317 (T090, an agonist for LXR α) induced
491 substantial lipid deposition in murine AML12 hepatocytes (Day 5). Right panel: lipids in
492 cytosol and nuclei stained with AdipoRed (green) and Hoechst33342 (red, pseudo-color),
493 respectively. Scale bar: 50 μm . Different letters indicate statistically significant differences
494 among groups ($p < 0.05$); $n = 4$; mean \pm SEM. (B) Cell growth deteriorated similarly in both
495 FA- and T090-induced steatotic hepatocytes. The same letter or no letter indicates that the
496 difference is not significant within each time-point group ($p < 0.05$); $n = 3-4$; mean \pm SEM).
497 (C) BEs promoted cell growth of steatotic hepatocytes induced by FA- and T090-
498 administration (left and right panels, respectively) with a peak concentration at 5 $\mu\text{g}/\text{mL}$.
499 Since no significant difference in cell growth was observed between 12 hours and 48 hours,
500 only the data after 60 hours are shown. The same letter or no letter indicates that the
501 difference is not significant within each time-point group ($p < 0.05$); $n = 3-4$; mean \pm SEM.

502

503 **Figure 2.** Bilberry fruits extracts (BEs) reduced lipid deposition in steatotic hepatocytes,
504 promoted cell growth, and conferred resistance to oxidative stress. (A) BEs significantly
505 suppressed lipid accumulation in hepatocytes. Right panel: lipids in cytosol and nuclei in
506 green (AdipoRed) and red (Hoechst33342, pseudo-color), respectively. Scale bar: 50 μm .
507 Different letters indicate statistically significant differences among groups ($p < 0.05$); $n = 4-6$;
508 mean \pm SEM. (B) BEs promoted cell growth at peak concentrations of 1-5 $\mu\text{g}/\text{mL}$ in
509 non-steatotic hepatocytes. The same letter indicates that the difference is not significant
510 ($p \geq 0.05$); $n=3-6$). (C) BEs conferred resistance to a 24-h H_2O_2 challenge (500 μM) in
511 hepatocytes. Lower panel: more live cells were observed in BEs-treated hepatocytes (1

512 $\mu\text{g/mL}$) than untreated liver cells. Scale: 50 μm . The same letter indicates that the difference is
513 not significant ($p \geq 0.05$); $n=3-6$). (D) BEs did not directly react with and/or scavenge cellular
514 ROS (left panel; $n = 8$).

515

516 **Figure 3.** Bilberry fruits extracts (BEs) improved liver steatosis, liver damage, and
517 hyperglycemia in mice fed HF&HC diets. (A) HF&HC diet (8 weeks) induced liver mass
518 enlargement with no increase in body weight. The same letter indicates that the difference is
519 not significant ($p \geq 0.05$); $n=4$. (B) BEs improved hepatic steatosis. Scale bar: 100 μm . Hepatic
520 TG levels were reduced in BEs-treated livers (right panel). The same letter indicates that the
521 difference is not significant ($p \geq 0.05$); $n=4$. (C) Fibrosis stained blue with Azan and was
522 observed in the periportal area (arrowhead) of HF&HC-treated livers. (D) Blood biochemistry
523 revealed that BEs improved steatosis-induced liver damage (ALT and AST) and
524 hypercholesterolemia (total cholesterol, T-CHO). The same letter indicates that the difference
525 is not significant within each group ($p \geq 0.05$); $n = 4$. Mean \pm SEM.

526

527 **Figure 4.** Molecular analyses of the effects of bilberry fruits extracts (BEs) on murine
528 AML12 hepatocytes. (A) Effects of BEs on autophagy (lipophagy) (Rubicon, p62/SQSTM1)
529 and fatty acid (FA) synthesis (fatty acid synthase, FASN) were examined in FA- / T0901317
530 (T090; LXR α agonist)-treated AML12 cells by western blot. Each blot represents ≥ 3
531 independent experiments. The duplicates of immunoblots are taken from the specimens of
532 experiments performed at different times. ImageJ (NIH, Bethesda, MD, USA) was used for
533 the quantitative analysis of western blot. The same letter indicates that the difference is not
534 significant among groups ($p \geq 0.05$). (B) Effects of BEs on FA synthesis were examined by
535 qRT-PCR. The same letter indicates that the difference is not significant among groups
536 ($p \geq 0.05$). (C) Levels of proteins associated with autophagy, survival/proliferation, and

537 antioxidant properties were slightly increased by the addition of BEs in non-steatotic AML12
538 cells (Day 3 and/or Day 7). Each blot represents ≥ 3 independent experiments. Each
539 experiment was performed 3 \times and the data are expressed as means \pm SEM (A, B, and C). The
540 same letter or no letter indicates that the difference is not significant within each time-point
541 group ($p \geq 0.05$).

542 **Supplemental data**

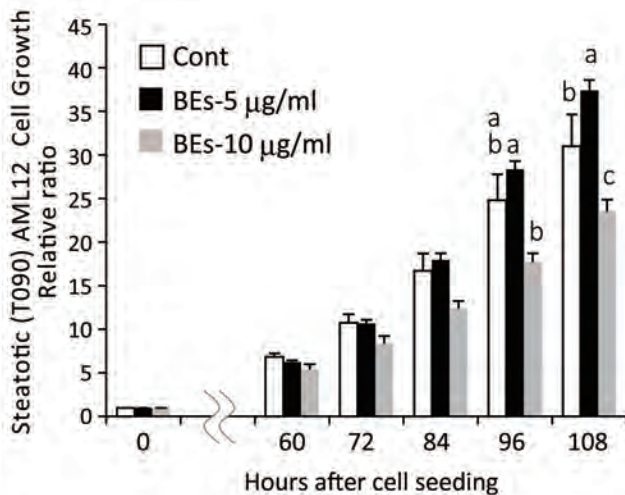
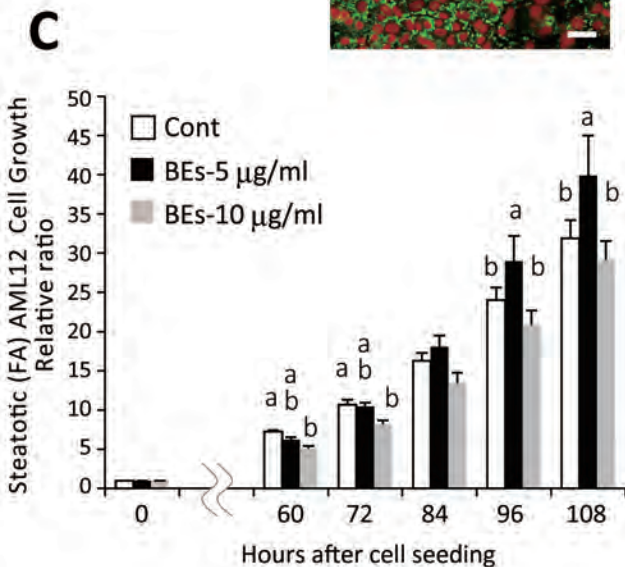
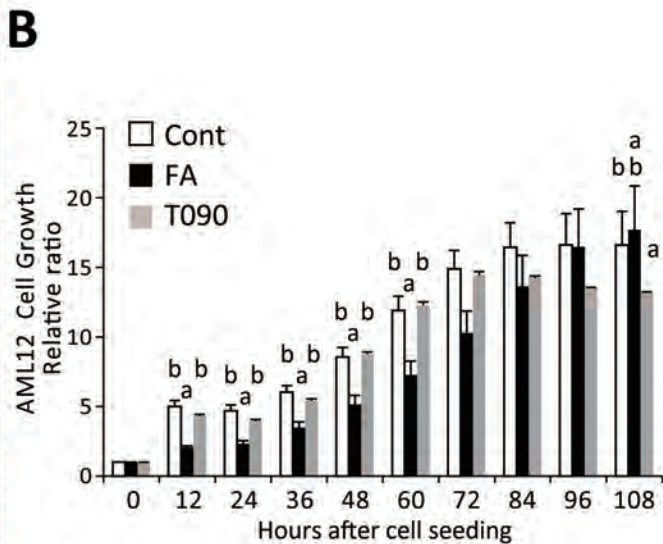
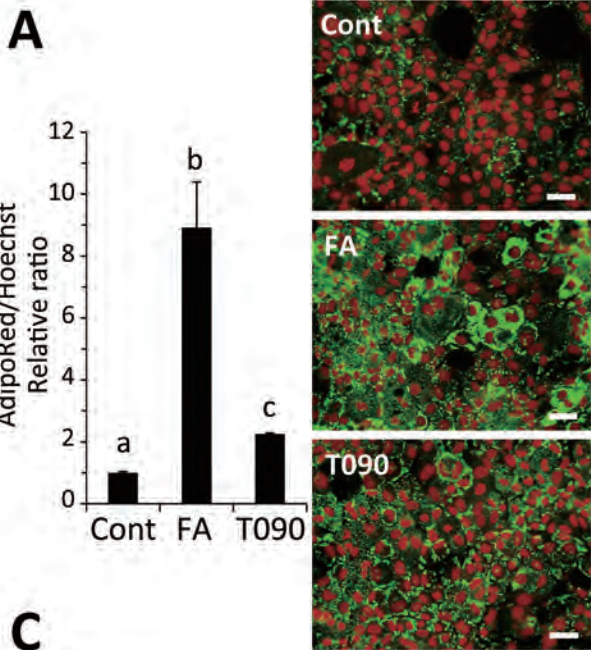
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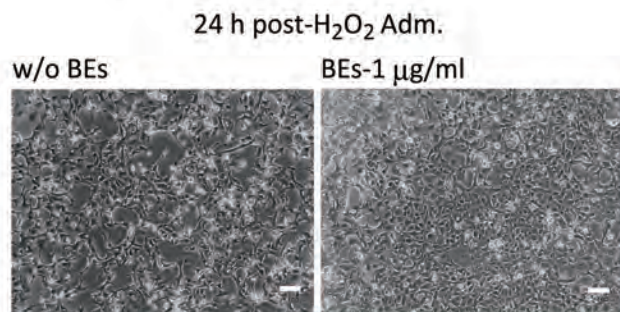
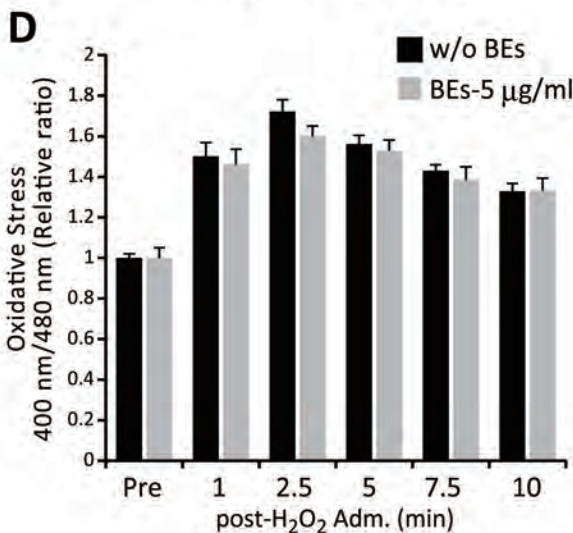
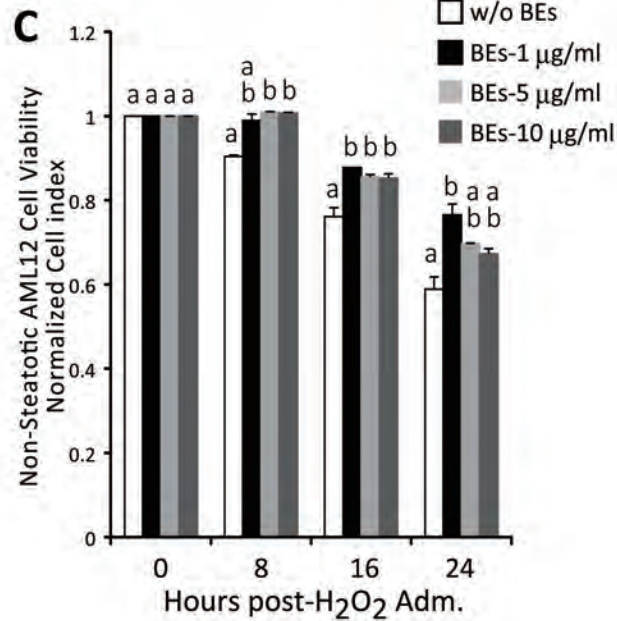
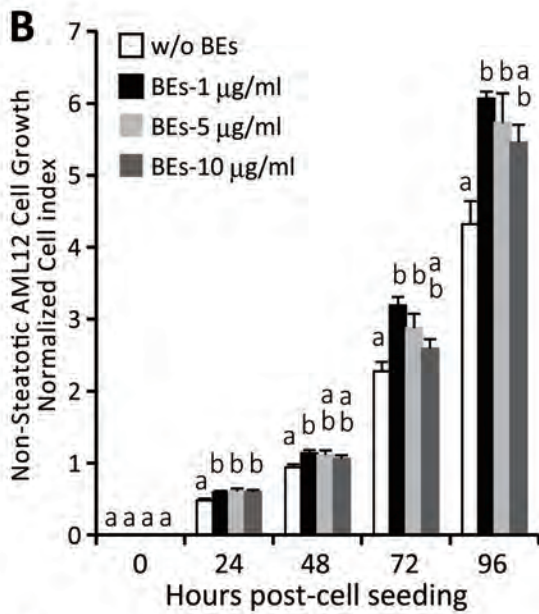
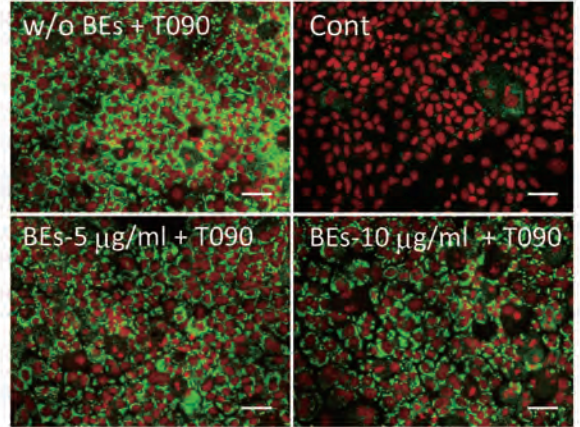
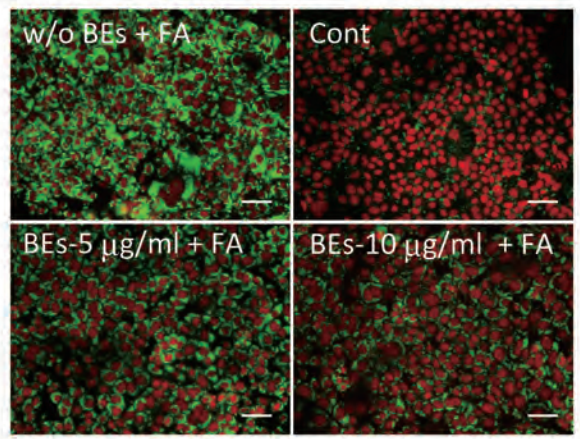
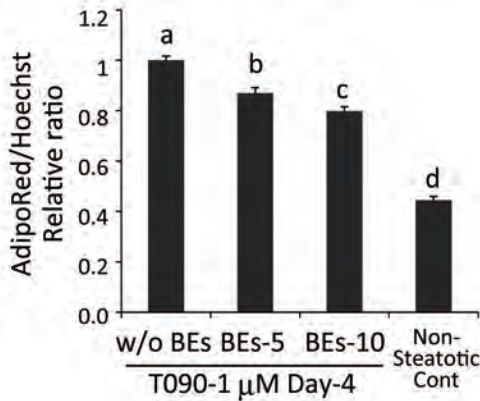
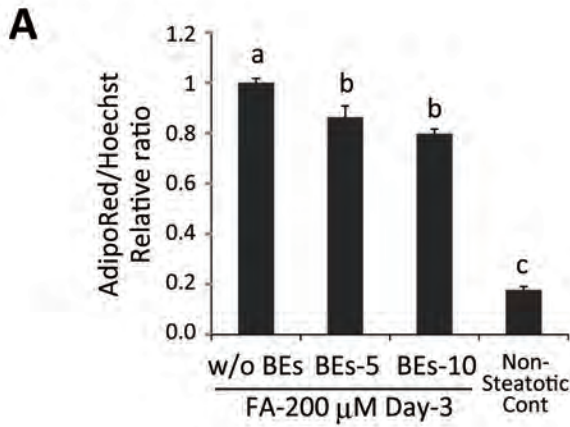
544 **Figure S1.** Cytotoxicity of bilberry fruits extracts (BEs) against AML12 cells estimated with
545 LDH assay. Values are expressed as means \pm SEM (n = 2).

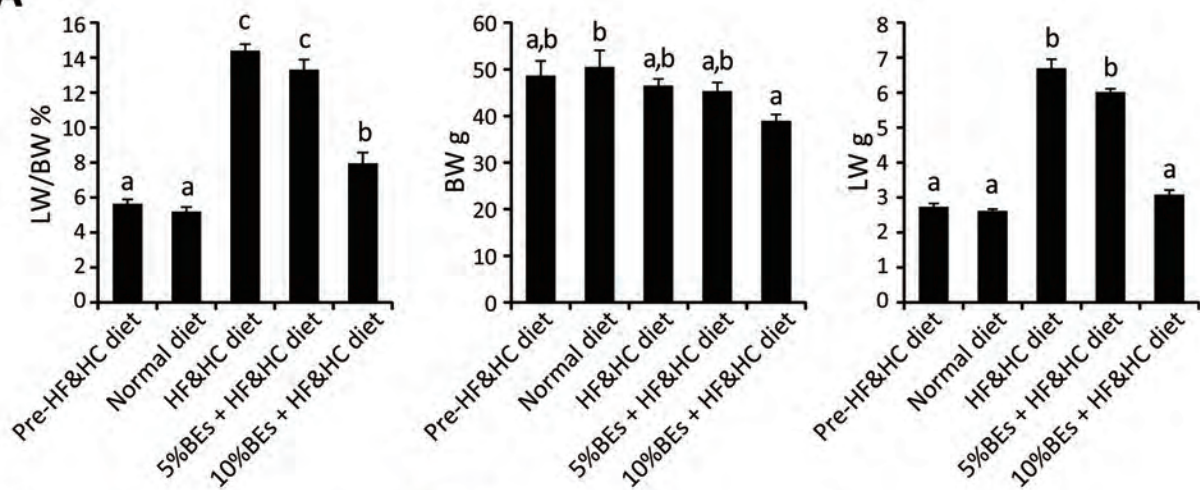
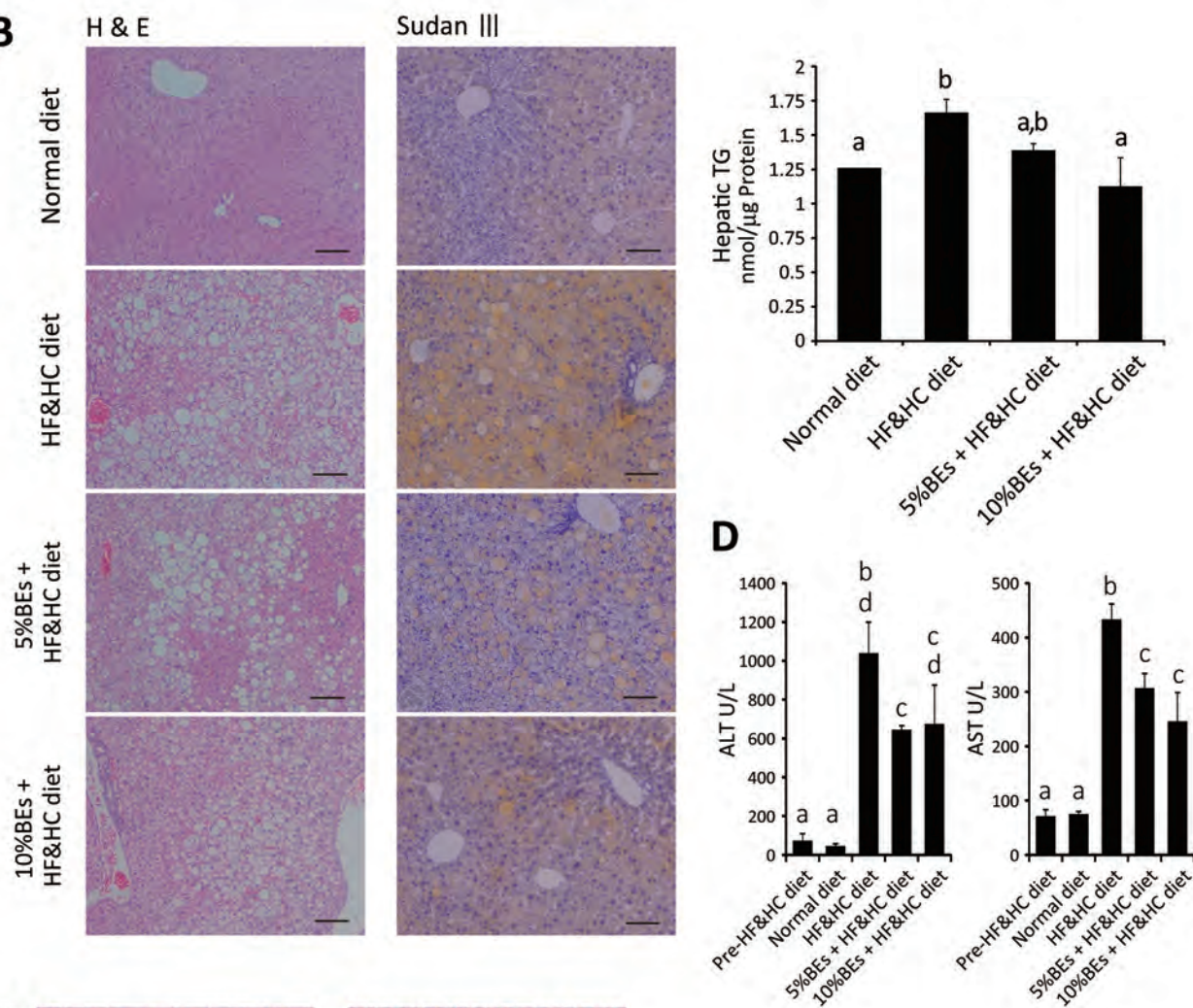
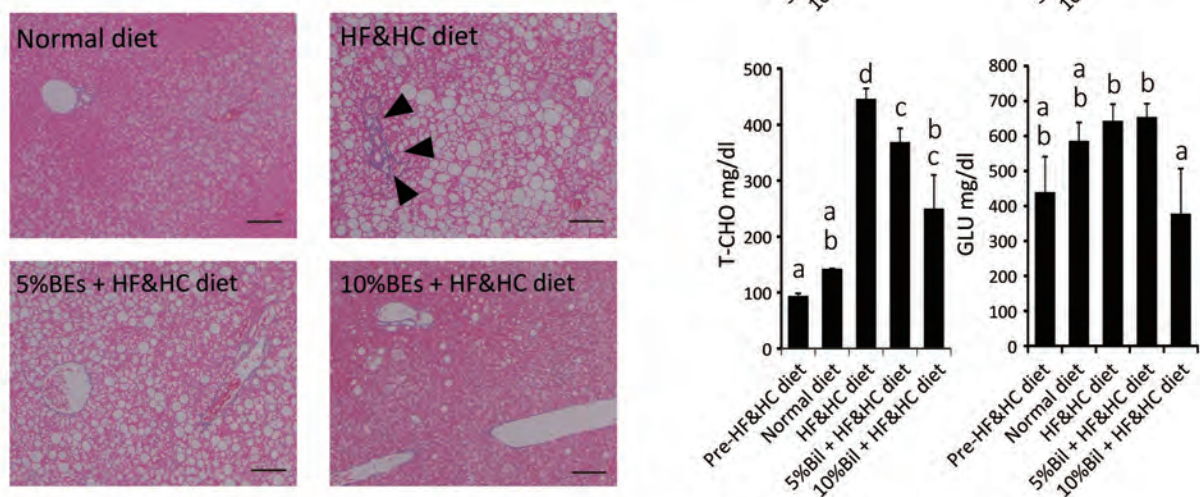
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547 **Figure S2.** Levels of pro-inflammatory cytokines induced by the high-fat and
548 high-cholesterol (HF&HC) diet decreased with bilberry fruits extracts (BEs) feeding.
549 Pro-inflammatory cytokines (Interleukin-1 β (IL-1 β), IL-9, IL-12, Interferon γ (IFN γ),
550 keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and tumor
551 necrosis factor α (TNF α)) in mouse plasma were measured with a Bio-Plex Multiplex System
552 (Bio-Rad Laboratories, Hercules, CA, USA). Values are expressed as means \pm SEM (n = 2-3).
553 There were no significant differences between the HF&HC diet group and the BEs + HF&HC
554 diet group.

555





A**B****C**

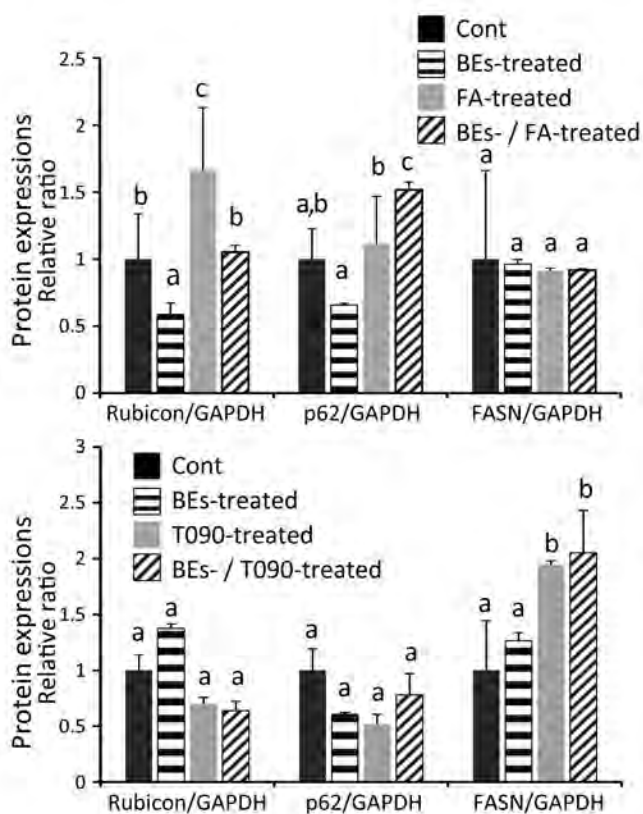
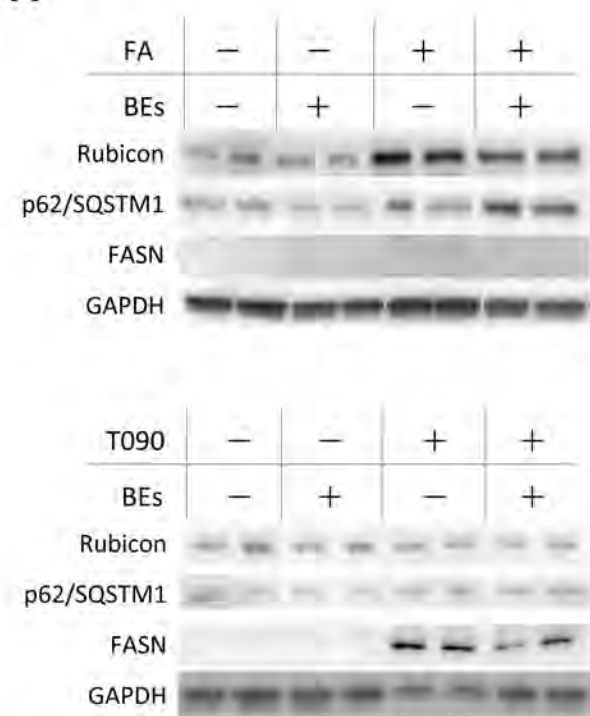
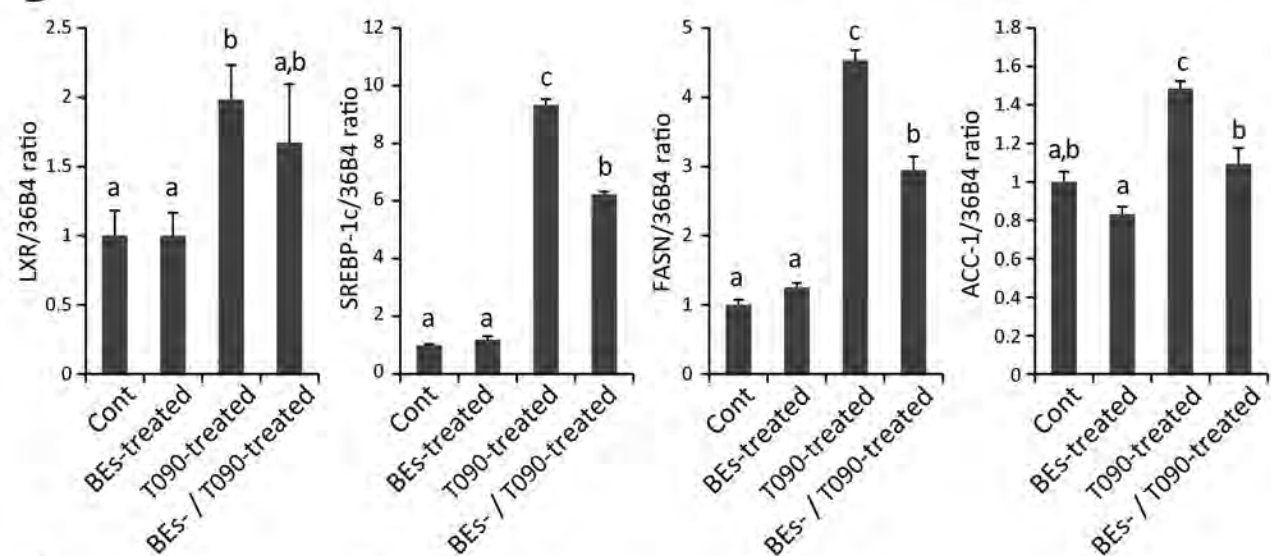
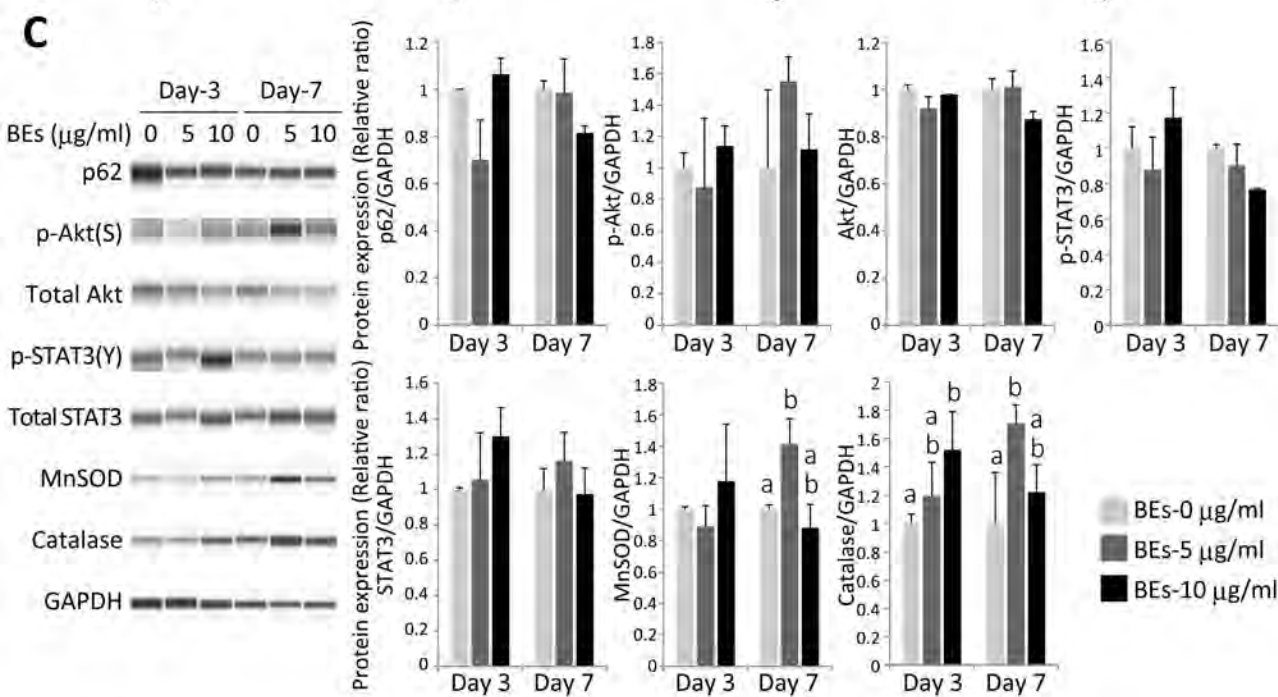
A**B****C**

Figure S1

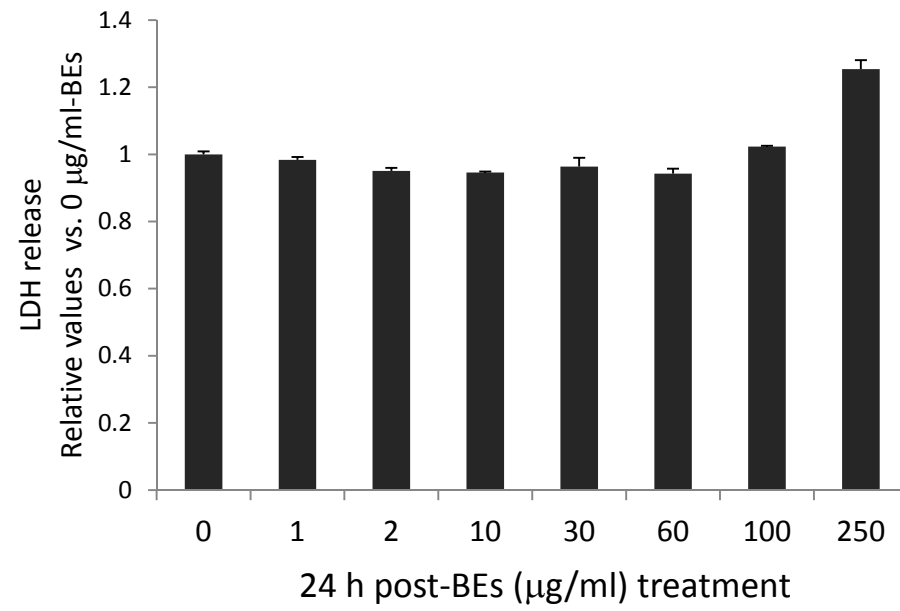


Figure S2

