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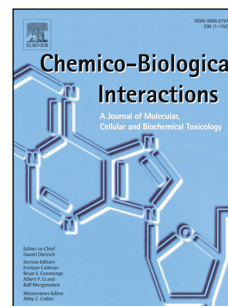

Protective effects of *Rubus tereticaulis* leaves ethanol extract on rats with ulcerative colitis and bio-guided isolation of its active compounds: A combined *in silico*, *in vitro* and *in vivo* study

Ali Şen, Dilek Özbeyli, Kerem Teralı, Fatih Göger, Aybeniz Yıldırım, Büşra Ertaş, Ahmet Doğan, Leyla Bitiş, Göksel Şener

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1 **Title page**

2 **Protective effects of *Rubus tereticaulis* leaves ethanol extract on rats with ulcerative colitis**
3 **and bio-guided isolation of its active compounds: A combined *in silico*, *in vitro* and *in vivo***
4 **study**

5

6 **Running title:** Bioactivity of *Rubus tereticaulis* and its major compounds

7

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38 **ABSTRACT**

39 The aim of this study was to evaluate the therapeutic effect of active ethanol extract obtained
40 from the leaves of *Rubus tereticaulis* (RTME) against colitis, and to purify major compounds
41 from this extract by bioassay-directed isolation. Rats with colitis induced via intra-rectal acetic
42 acid administration (5%, v/v) received RTME or sulfasalazine for three consecutive days. On
43 day four, all rats were decapitated, and the colonic tissue samples were collected for
44 macroscopic score, colon weight, reduced glutathione (GSH), myeloperoxidase (MPO), and
45 malondialdehyde (MDA) analyses. The active compounds and chemical composition of RTME
46 were determined by bio-guided isolation and LC-MS/MS, respectively. Compared to the colitis
47 group, the rats treated with RTME displayed significantly lowered macroscopic scores and
48 colon wet weights ($p<0.001$). These effects were confirmed biochemically by a decrease in
49 colonic MPO activity ($p<0.001$), MDA levels ($p<0.001$), and an increase in GSH levels
50 ($p<0.001$). Kaempferol-3-*O*-β-D-glucuronide (RT1) and quercetin-3-*O*-β-D-glucuronide (RT2)
51 were found to be the major compounds of RTME, as evidenced by *in vitro* anti-inflammatory
52 and antioxidant activity-guided isolation. Their anti-inflammatory/antioxidant activities were
53 also predicted by docking simulations. Additionally, quinic acid, 5-caffeoylquinic acid,
54 quercetin pentoside, quercetin glucoside, quercetin-3-*O*-β-D-glucuronide, kaempferol-3-*O*-β-D-
55 glucuronide, and kaempferol rutinoside were identified in RTME via using LC-MS/MS. RT2,
56 along with other compounds, may be responsible for the observed protective action of RTME
57 against colitis. This study represents the first report on the beneficial effects of RTME in an
58 experimental model of colitis and highlights the potential future use of RTME as a natural
59 alternative to alleviate colitis.

60

61 **Keywords:** Antioxidant activity, anti-inflammatory activity, colitis, flavonoids, *Rubus*
62 *tereticaulis*

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64 **1. Introduction**

65 Ulcerative colitis (UC) is a persistent type of inflammatory bowel disease (IBD) that affects the
66 colon mucosa. UC is largely associated with oxidative stress and inflammation that cause
67 damage to colon tissue [1].

68 Oxidative stress and inflammation form the basis of the pathogenesis of many diseases.
69 Free radicals produced by the physical and biochemical processes that occur in the human body
70 damage various biomolecules that are important for the body, thereby causing various diseases
71 [2]. Inflammation is an indeterminate physiological response of the body to damage caused by
72 endogenous or exogenous agents, and is an inevitable limited response of the body to
73 spontaneous exposure when the tissue is exposed to chemical, mechanical or biological damage.
74 Therefore, inflammation acts as one of the defense barriers of the organism. Keeping
75 inflammation under control plays an important role in the treatment of chronic inflammation-
76 related diseases such as arthritis, osteoarthritis, sclerosis, arteriosclerosis, Alzheimer's, diabetes,
77 insulin-resistance, obesity, allergies, asthma, chronic bronchitis, cancer, tuberculosis, retinitis,
78 psoriasis, lung fibrosis, and chronic gastritis [3].

79 Reactive oxygen species (ROS) are signaling molecules that play an important role in
80 the progression of inflammatory diseases [4]. Antioxidants reduce health risks associated with
81 oxidative stress caused by intense ROS such as free radicals. It has been found that certain
82 polyphenol-based diets, including polyphenols that are potent antioxidant and anti-
83 inflammatory agents, have therapeutic efficacy in reducing inflammation and oxidative stress
84 [5]. Furthermore, phenolic compounds and flavonoids present in plants are known to have
85 antioxidant, anti-cancer, antimicrobial, antiviral and anti-inflammatory activities [6]. As a
86 result, it is crucially important to conduct research on medicinal plants and their polyphenolic-
87 rich extracts.

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88 Although aminosalicylic acid, corticosteroids, and thiopurines used for the prevention
89 and treatment of IBD today have somewhat improved the quality of life of patients with IBD,
90 they have not been effective at all stages of the disease [7]. Therefore, new drugs with less side
91 effects than those currently used are needed.

92 The genus *Rubus*, a member of the Rosaceae family, is represented by naturally grown
93 10 taxa in Turkey [8]. *Rubus* species including *Rubus tereticaulis* in various regions of Turkey
94 in traditional medicine are generally used to treat wounds, cuts and burns as well as skin
95 diseases like eczema and psoriasis [9]. In addition, different parts of the *Rubus* species (fruit,
96 leaves, and roots) are used in the treatment of kidney and prostate inflammation, hemorrhoid,
97 diarrhea, inflammatory small bowel diseases, diabetes mellitus, rheumatism, sore throat, colds,
98 influenza, and cardiovascular diseases, also as an immune system booster and hemostatic
99 [10,11]. In particular, it was noted in the literature that the leaves of *Rubus tereticaulis* are used
100 by the public for anti-inflammatory purposes [12].

101 Scientific studies on *Rubus* species have revealed that these species harbor
102 antimicrobial, radical scavenging, anticonvulsant, muscle relaxant, anti-inflammatory and
103 antinociceptive activities [11]. Phytochemical analyses of *Rubus* species have indicated the
104 presence of flavonoids (quercetin, kaempferol, etc.) and phenolic acids (caffeic acid,
105 chlorogenic acid, etc.). Additionally, the presence of catechins, pectins, carboxylic acids,
106 anthocyanins, vitamin C, and saturated or unsaturated fatty acids has also been demonstrated
107 by earlier reports [13,14]. To the best of our knowledge, there is no scientific information
108 regarding the chemical composition and biological activity of *Rubus tereticaulis* leaves.
109 Therefore, the aim of this study was to investigate the protective effects of *Rubus tereticaulis*
110 leaves ethanol extract (RTME) on acetic acid-induced colitis in rats and obtain the active
111 compounds from RTME through *in vitro* anti-inflammatory and antioxidant activity by
112 bioassay-directed isolation.

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113 **2. Materials and methods**

114 *2.1. Plant material*

115 Leaves of *Rubus tereticaulis* were collected in the flowering period from Sile district of Istanbul
116 province of Turkey on June 15, 2016 and identified by Dr. Ahmet Dogan. Voucher specimens
117 were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No:
118 18573).

119

120 *2.2. Extraction*

121 Dried and ground leaves of *Rubus tereticaulis* (10 g) for *in vitro* activities were extracted with
122 96% EtOH (3 × 100 mL) at room temperature, using maceration methods. After filtration and
123 evaporation, the obtained ethanol extract was dissolved in 50 mL of 50% aqueous ethanol and
124 subjected to solvent–solvent partition between *n*-hexane (3 × 50 mL), chloroform (3 × 50 mL)
125 and ethyl acetate (3 × 50 mL). The ethanol extract obtained by maceration and its *n*-hexane,
126 chloroform, ethyl acetate and aqueous ethanol fractions were coded as RTME, RTMH, RTMC,
127 RTMEA and RTMAE, respectively. Also, about 300 g of the plant was weighed for isolation
128 and *in vivo* studies, and similar extraction procedures were applied as described above. Percent
129 yields of all extracts (Table 2) were calculated, and all extracts were stored under refrigeration
130 for further analysis.

131

132 *2.3. Determination of in vitro anti-inflammatory activity*

133 The anti-LOX activity was evaluated as described by Phosrithong and Nuchtavorn [2]. 500 µL
134 of extracts, isolated compounds and standard indomethacin were added to 250 µL of sodium
135 borate buffer solution (0.1 M, pH 9) followed by addition of 250 µL of type V soybean LOX
136 solution in buffer (pH 9, 20.000 U/mL). The mixture was preincubated at 25 °C for 5 min. Then,
137 1000 µL of 0.6 mM linoleic acid solution was added, mixed well and the change in absorbance

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138 at 234 nm was recorded for 6 min. The percent inhibition was calculated from the following
139 equation:

140 $$\% \text{ inhibition} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

141 A dose–response curve was plotted to determine the IC_{50} values. IC_{50} was defined as the
142 concentration sufficient to obtain 50% of maximum anti-inflammatory activity. Tests were
143 carried out in triplicates. Indomethacin was used as a positive control.

144

145 *2.4. Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity*

146 Free radical scavenging capacity of samples was measured according to a previously reported
147 procedure [15]. Briefly, 10 µL of samples in DMSO at different concentrations (250–0.49
148 µg/mL) were mixed with 190 µL of 0.1 mM DPPH solution in MeOH in wells of a 96-well
149 plate. Mixtures were shaken vigorously and left to incubate for 30 min in the dark at room
150 temperature. Then, the absorbance was read at 517 nm. The percent radical scavenging activity
151 of extracts and compounds against DPPH radical was calculated according to the following
152 equation:

153 $$\text{DPPH radical-scavenging activity } (\%) = [(A_0 - A_1) / A_0] \times 100$$

154 where A_0 is the absorbance of the control (containing all reagents except the test
155 extracts/compounds) and A_1 is the absorbance of the extracts/compounds. The
156 extract/compound concentration that produced 50% inhibition (IC_{50}) was calculated from the
157 graph, plotting inhibition percentage versus extract concentration. All tests were carried out in
158 triplicates. Ascorbic acid and butylated hydroxytoluene were used as positive controls.

159

160

161

162 *2.5. Determination of 2.2'-azinobis (3-ethylbenzothiazolin-6-sulphonic acid (ABTS) radical-*
163 *scavenging activity*

164 ABTS radical cation scavenging activity assay was tested according to [15]. ABTS radical
165 cations were produced by mixing equal volumes of ABTS (7 mM in H_2O) and potassium
166 persulfate (4.9 mM in H_2O), allowing them to react for 12–16 h at room temperature in the
167 dark. The ABTS radical solution was then diluted by mixing it with 96% ethanol to obtain an
168 absorbance of about 0.7 at 734 nm using a spectrophotometer. 10 µL of samples in DMSO at
169 different concentrations (250–0.49 µg/mL) were added to 190 µL of ABTS radical solution in
170 a 96-well microplate. The mixture was incubated at room temperature in the dark for 30 min.
171 Then, absorbance readings were taken at 734 nm. The percent radical scavenging activity of
172 the extracts and compounds against ABTS radical was calculated according to the following
173 equation:

174 $$\text{ABTS radical-scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

175 where A_0 is the absorbance of the control (containing all reagents except the test
176 extracts/compounds) and A_1 is the absorbance of the extracts/compounds. The
177 extract/compound concentration that produced 50% inhibition (IC_{50}) was calculated from the
178 graph, plotting inhibition percentage versus extract concentration. All tests were carried out in
179 triplicates. Trolox and butylated hydroxytoluene were used as positive controls.

180

181 *2.6. Animals and experimental design*

182 Wistar albino rats (250–300 g) were supplied by the Marmara University (MU) Application
183 and Research Center for Experimental Animals (DEHAMER). Rats were housed in an air-
184 conditioned room with 12:12 light:dark cycles, where the temperature (22 ± 2 °C) and relative
185 humidity (65–70%) were kept constant. All experimental protocols were approved by the MU
186 Animal Care and Use Committee (Protocol number: 51.2020. mar). The rats were randomly

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187 divided into five groups, with six rats in each group: control group; 300 mg/kg *Rubus* group;
188 colitis (AA)+physiological saline group; colitis+300 mg/kg *Rubus* group; and colitis+100
189 mg/kg sulfasalazine (SS) group. All sets of treatment regimens were given by oral gavage and
190 administered as a single dose daily. Treatment was started following colitis induction and
191 continued for 3 consecutive days.

192

193 *2.7. Induction of ulcerative colitis*

194 After 8 hours of fasting, 1 mL of 5% (v/v) AA in 0.9% NaCl was administered intrarectally to
195 rats under mild ether anesthesia with an 8-cm-long cannula [16]. The rats in the control group
196 were given 0.9% NaCl in the same volume and in the same way.

197

198 *2.8. Assessment of colitis severity*

199 All rats were euthanized under ketamine anesthesia on the fourth day after colitis induction.
200 The distal 8 cm of the colon were opened longitudinally, the lumen contents were cleared, rinsed
201 in saline and dried on a filter paper. Macroscopic scoring was performed using the criteria
202 outlined in Table 1 for the evaluation of mucosal lesions [17,18].

203

204 **Table 1.** Criteria for macroscopic scoring of colonic lesions

Score	Appearance
0	No damage
1	Localized hyperemia, no ulcers
2	Ulceration without hyperemia or bowel wall thickening
3	Ulceration with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Major sites of damage extending more than 1 cm along the length of colon
6–10	If damage extends more than 2 cm along the length of colon, the score is increased by one for each additional 1 cm

205

206

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208 *2.9. Measurement of tissue MDA and GSH levels*

209 For the determination of MDA and GSH levels, collected colonic samples were homogenized
210 with ice-cold 150 mM KCl. Lipid peroxidation was expressed in terms of MDA equivalents
211 using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}, and results were expressed as nmol
212 MDA/g tissue [19]. GSH measurements were performed using a modification of the Ellman's
213 procedure [20]. GSH levels were calculated using an extinction coefficient of 1.36×10^4 M^{-1}
214 cm^{-1}. Results were expressed in mmol GSH/g tissue.

215

216 *2.10. Measurement of tissue MPO activity*

217 Colonic MPO activity was measured with H_2O_2-dependent oxidation of o-Dianisidine
218 dihydrochloride at 37 °C. One unit of enzyme activity was defined as the amount of MPO
219 present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was
220 expressed in U/g tissue [21].

221

222 *2.11. Isolation of active compounds from RTME*

223 In *in vitro* bioactivity experiments, RTMEA showed the best antioxidant and anti-LOX activity
224 among all fractions of RTME. Therefore, RTMEA was chosen for further isolation
225 experiments. RTMEA (2.0853 g) was fractionated by CC on Sephadex, using CH_3OH to yield
226 forty-two fractions. Fractions exhibiting similar TLC profiles (TLC silica gel 60 F_{254} plates were
227 developed using 8:1:1 ethyl acetate:formic acid:water as eluent) were combined to give four
228 sub-fractions (F1-F6: 0.4996 g; F7-F15: 0.5599 g; F16-F27: 0.2827 g; F28-F42: 0,2294 g).
229 DPPH and anti-inflammatory activity tests were performed utilizing these fractions. We
230 continued the isolation with F7-F15, having the highest activity and the most intense compound
231 content on TLC among all fractions, F7-F15 (0.5537 g) was repeatedly subjected to size-
232 exclusion chromatography on a Sephadex LH-20 column, eluted with CH_3OH and then

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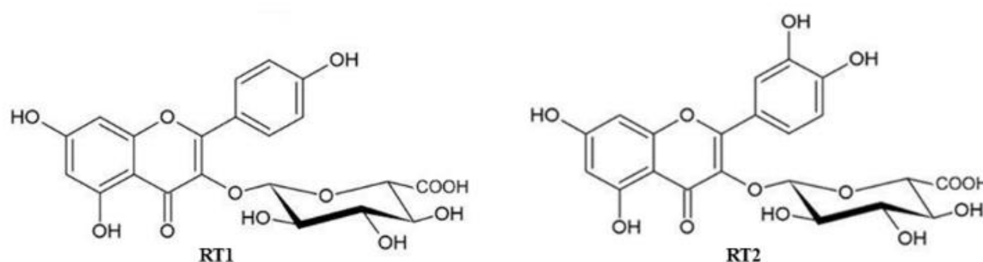
233 combined sub-fractions was re-chromatographed by preparative TLC with ethyl acetate:formic
234 acid:water (8:1:1) to give RT1 (30.3 mg) and RT2 (23.4 mg) (Figure 1)



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236 **Figure 1.** Chemical structures of major compounds isolated from *R. tereticaulis*

237

238 *2.12. LC-MS/MS analysis of RTME*

239 Absciex 3200 MS/MS detector was used for the LC-MS/MS analysis. Negative ionization mode
240 was selected for ionization. Chromatographic separations were achieved with ODS C-18 250 ×
241 4.6 mm, i.d., 5 µm column using the Shimadzu 20A HPLC system. The column oven
242 temperature was set at 40 °C, and the flow rate was adjusted to 0.5 mL/min. Mobile phases (A)
243 acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1,
244 v/v/v). The concentration of B was increased from 10% to 100% in 40 minutes. For mass
245 scanning (EMS), a mass range of 100–1000 amu was chosen.

246

247 *2.13. Determination of the total phenolic contents of Rubus tereticaulis extract and its fractions*

248 The total phenolic compound content of extracts was determined according to Gao et al. [22].
249 The assay was adapted to the 96-well microplate format [23]. 10 µL of extracts in various
250 concentrations were mixed with 20 µL of the Folin-Ciocalteu reagent (Sigma), 200 µL of H_2O,
251 and 100 µL of 15% Na_2CO_3. After 2 h incubation at room temperature, absorbance was read at
252 765 nm. Gallic acid was used as the standard compound, and the total phenolic amounts of
253 extracts were expressed in mg/g gallic acid equivalents (GAE).

254

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255 *2.14. Determination of the total flavonoid contents of Rubus tereticaulis extract and its fractions*

256 The total flavonoid compound content of extracts was determined according to Zhang et al.
257 [24]. The assay was adapted to the 96-well microplate format [23]. 25 µL of extracts in various
258 concentrations were mixed with 125 µL of H_2O and 7.5 µL of 5% $NaNO_2$. After 6 min, 15 µL
259 of 10% $AlCl_3$ solution was added and incubated for 5 min, followed by the addition of 50 µL
260 of 1 M NaOH solution. Distilled water was added to bring the total volume to 250 µL, and the
261 absorbance was immediately read at 510 nm. Catechin was used as standard, and total flavonoid
262 content was expressed in mg CE/g of dry weight of extract.

263

264 *2.15. Protein–ligand docking and interaction profiling*

265 The SMILES strings of RT1 and RT2 were translated into energy-minimized 3D structures with
266 appropriate topologies and parameters by using the myPresto program suite (available at
267 https://demo1.biomodeling.co.jp/). The 3D structures of COX-2 (PDB ID: 3LN1; [25]) and
268 iNOS (PDB ID: 3E7G; [26]) in complex with selective small-molecule inhibitors were retrieved
269 from the RCSB Protein Data Bank ([37]; available at https://www.rcsb.org/). Protein–ligand
270 docking was achieved with high precision by using JAMDA (available at https://proteins.plus/)
271 that combines the TrixX docking algorithm [28,29] with the JAMDA scoring function [30].
272 The protein was prepared by keeping all heteroatoms (except those of the reference ligand and
273 water molecules within the binding site), and the most likely protonation states and hydrogen
274 coordinates were assigned to the protein through Protoss optimization. The binding site was
275 defined by the reference ligand, with a site radius of 6.5 Å. The docking poses generated were
276 prioritized based on their JAMDA scores. Favorable non-covalent interactions between the
277 proteins and ligands were computed by using Discovery Studio v16.1.0 (Dassault Systèmes
278 BIOVIA Corp., USA).

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280 *2.16. Statistical analysis*

281 Statistical analyses were performed by using the InStat statistical analysis package (GraphPad
282 Software, San Diego, CA). Data were expressed as the mean ± SEM. Biochemical data were
283 compared by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.
284 *P*-values less than 0.001 and 0.05 were considered statistically significant.

285

286 **3. Results**

287 *3.1. Colonic injury severity, malondialdehyde (MDA) and glutathione (GSH) levels,*
288 *myeloperoxidase (MPO) activity*

289 Based on our observations as well as on the well-established ethnobotanical use of the plant,
290 we were interested in exploring the potential positive effects of RTME on acetic acid-induced
291 UC in rats. We demonstrated, in the first instance, that acetic acid–induced colitis caused a
292 significant increase in the macroscopic score, colon wet weight, colonic MPO activity and
293 MDA level as well as a significant decrease in GSH levels in colon tissues compared to the
294 control group ($p<0.001$) (Figures 2–4) The macroscopic score, colon wet weight, colonic MPO
295 activity and MDA levels in the group treated with RTME displayed a significant reduction
296 compared to the colitis group ($p<0.001$) (Figures 2–4). Also, a significant increase in GSH
297 levels was observed in colon tissue of the group treated with RTME ($p<0.001$) (Figure 4). Effect
298 of RTME on colon ulcer induced with acetic acid was almost similar to the sulfasalazine group
299 (Figures 2–4).

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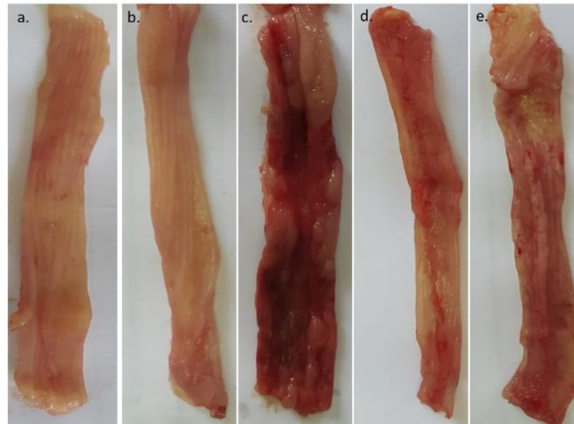
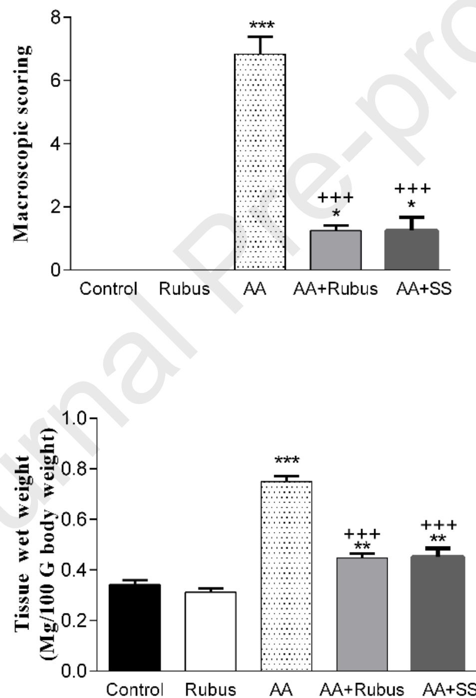


300

301 **Figure 2.** Macroscopic appearance of colonic tissues of **a)** control group, **b)** 300 mg/kg *Rubus* group, **c)** colitis
302 (AA)+physiological saline group, **d)** colitis+300 mg/kg *Rubus* group, **e)** colitis+100 mg/kg sulfasalazine (SS)
303 group.



304

305 **Figure 3.** Macroscopic lesion scores and wet weight of colonic tissues of control group, 300 mg/kg *Rubus* group,
306 colitis (AA)+physiological saline group, colitis+300 mg/kg *Rubus* group, colitis+100 mg/kg sulfasalazine (SS)
307 group. Values are represented as mean ± sem. *$p<0.05$, **$p<0.01$, ***$p<0.001$; versus control group. $^{+++}p<0.001$;
308 versus ps-treated colitis group.

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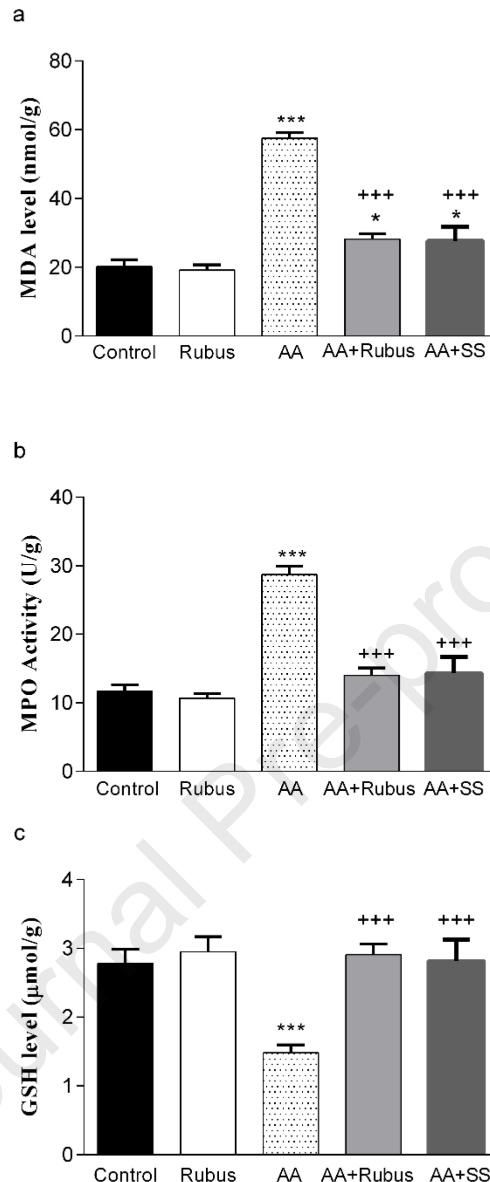

309

310 **Figure 4.** Malondialdehyde (MDA) levels, myeloperoxidase (MPO) activities and glutathione (GSH) levels in the
311 colonic tissues of control group, 300 mg/kg *Rubus* group, colitis (AA)+physiological saline group, colitis+300
312 mg/kg *Rubus* group, colitis+100 mg/kg sulfasalazine (SS) group. Values are represented as mean ± sem. *p<0.05,
313 ***p<0.001; versus control group. ^{+++}p<0.001; versus ps-treated colitis group.

314

315 *3.2. In vitro antioxidant and anti-inflammatory activity of RTME*

316 The results of the *in vitro* tests showed that RTME had strong antioxidant activity, with
317 IC_{50} values of 18.31 μg/mL and 13.91 μg/mL against DPPH and ABTS radicals, respectively.
318 RTME exhibited significant anti-inflammatory activity by inhibiting the development of LOX
319 enzyme activity by 91.15% at a concentration of 156 μg/mL (Table 2). Also, the amounts of

320 total phenolics and flavonoids of RTME were found to be 35.55 and 6.94 mg/g , respectively
321 (Table 3).

322 **Table 2.** The yields and antioxidant/anti-inflammatory activities of extracts obtained from the
323 leaves of *R. tereticaulis*
324

Extracts*	**Yields**	**ABTS activity IC_{50} (µgmL^{-1})****	**DPPH activity IC_{50} (µgmL^{-1})****	**Anti-inflammatory activity (% inhibition at 156 µgmL^{-1})**
RTME	15.74	18.31±0,18^{b}	13.91±0,17^{b}	91.15±0.00^{d}
RTMH	1.51	507.6±5,15^{d}	884.5±5,20^{d}	37.31±0.06^{a}
RTMC	2.25	27.10±2,72^{c}	34.79±0,06^{c}	57.35±0.0,15^{b}
RTMEA	2.95	12.34±0,52^{a}	9.00±0,87^{a}	99.01±0,11^{e}
RTMAE	8.35	18.27±0,20^{b}	12.49±0,39^{b}	74.88±0,06^{c}

325
326
327 * The ethanol extract and its n-hexane, chloroform, ethyl acetate and aqueous ethanol fractions obtained by maceration and
328 liquid–liquid extraction were coded as RTME, RTMH, RTMC, RTMEA and RTMAE, respectively. The yields of RTME was
329 calculated from the powdered dry plant. The yields of the remaining extracts were calculated from dried RTME.
330 ** IC_{50}: Values corresponding to the concentration of extract required to scavenge/inhibit 50% of radicals/enzyme present in
331 the reaction mixture.
332 *** Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate
333 significant differences ($p < 0.05$).
334
335

336 **Table 3.** Total phenolic and flavonoid contents of extracts obtained from the leaves of *R.*
337 *tereticaulis*
338

Extracts*	**TPC** (mg GAE/g extract)**	**TFC*** (mg CE/g extract)**
RTME	35.55±0,08^{c}	6.94±0,36^{c}
RTMH	3.53±0,30^{a}	4.162±0,06^{b}
RTMC	14.18±0,90^{b}	2.44±0,07^{a}
RTMEA	63.30±0,75^{d}	12.76±0,07^{d}
RTMAE	38.29±0,26^{c}	2.63±0,16^{a}

339
340 * The ethanol extract and its n-hexane, chloroform, ethyl acetate and aqueous ethanol fractions obtained by maceration were
341 coded as RTME, RTMH, RTMC, RTMEA and RTMAE, respectively.
342 ** Total phenolic content (TPC) was expressed as gallic acid equivalent (GAE).
343 *** Total flavonoid content (TFC) was expressed as catechin equivalent (CE).
344 **** Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate
345 significant differences ($p < 0.05$).
346
347

348 *3.3. Bio-guided isolation of potential antioxidant and anti-inflammatory compounds from*
349 *RTME*

350 RTME demonstrated significant bioactivity in both *in vitro* and *in vivo* assays.
351 Therefore, in an attempt to reveal the compounds that were responsible for this behavior, we
352 decided to perform bioassay-directed fractionation and subsequent isolation. Firstly, RTMH,
353 RTMC, RTMEA and RTMAE fractions were obtained by liquid–liquid extraction from RTME.
354 Then, *in vitro* antioxidant and anti-inflammatory activities of these fractions as well as total

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355 phenol and flavonoid contents were evaluated. RTMEA with IC_{50} values 12.34 and 9.00 µg/mL
356 against ABTS and DPPH radicals showed significant antioxidant activity when compared to
357 other fractions ($p<0.05$) (Table 2). Also, this fraction exhibited the highest anti-inflammatory
358 activity against 5-lipoxygenase at concentration of 156 µg/mL ($p<0.05$) (Table 2). In addition,
359 RTMEA had the most significant ($p<0.05$) amount of phenolic (63.30 mg/g) and flavonoid
360 (12.76 mg/g) compared to the other fractions (Table 3). Based on these results, priority was
361 given to the ethyl acetate fraction of RTME (RTMEA) for active compound isolation. Of the
362 obtained 4 sub-fractions (F1-F6; F7-F15; F16-F27; F28-F42), the isolation process was
363 continued with F7-F15, which showed the best activity. [DPPH radical and LOX inhibition rate
364 at a concentration of 50 µg/mL: 50.50 and 19.77% (F1-F6), 82.86 and 96.05% (F7-F15), 82.03
365 and 72.01% (F16-F27), 82.03 and 76.02% (F28-F42), respectively]. Two flavonol
366 glucuronides, kaempferol-3-*O*-*β*-D-glucuronide (RT1, 30.3 mg) and quercetin-3-*O*-*β*-D-
367 glucuronide (RT2, 23.4 mg), were isolated as major compounds from the active F7-F15 sub-
368 fraction of RTMEA (Figure 1). All isolated compounds were analyzed by spectroscopic
369 methods (UV, ^{1}H NMR, and ^{13}C NMR-APT), and the resulting data were compared with those
370 reported in the literature. (Detailed spectral data is included in supporting information.)

371

372 *3.4.* In vitro *antioxidant and anti-inflammatory activity of major compounds isolated from*
373 *RTME*

374 The *in vitro* antioxidant activities of the compounds were evaluated according to the
375 classification criteria described by Ervina et al. [31]. Normal classification of a compound's
376 antioxidant activity generally follows the followings criteria: a very powerful antioxidant (IC_{50}:
377 <50 µg/mL); a strong antioxidant (IC_{50}: 50–100 µg/mL); an intermediate antioxidant (IC_{50}:
378 101–150 µg/mL); and a weak antioxidant (IC_{50}: 151–200 µg/mL) [36]. When the antioxidant
379 activities of RT1 and RT2, which are the major compounds isolated from active ethyl acetate

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380 fraction of RTME, were evaluated, especially RT2 was found to exhibit significant antioxidant
381 activity against DPPH and ABTS radicals with IC_{50} values of 7.71 and 8.69 µg/mL,
382 respectively. Furthermore, RT1 with an IC_{50} of 49.14 µg/mL showed better anti-LOX activity
383 than RT2 (IC_{50}: 67.64 µg/mL) (Table 4).

384 **Table 4.** Antioxidant and anti-inflammatory activities of major compounds isolated from the
385 RTME
386

Compounds*	DPPH activity	ABTS activity	Anti-inflammatory activity
	IC_{50} (µgmL^{-1})**		
RT1	199.7±2,20^{d}	135.6±0,30^{d}	49.14±2.17^{b}
RT2	7.71±0.15^{b}	8.69±0,19^{b}	67.64±1.19^{c}
Ascorbic acid	2.5±0,18^{a}		
Trolox		3.17±0.00^{a}	
Butylated hydroxytoluene	57.15±0.00^{c}	17.06±0.00^{c}	
Indometacine			18.05±0,59^{a}

387 * Kaempferol-3-*O*-*β*-D-glucuronide and quercetin-3-*O*-*β*-D-glucuronide were coded as RT1 and RT2, respectively.
388 ** Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate
389 significant differences ($p < 0.05$).
390

391

392 *3.5. LC-MS/MS analysis of RTME*

393 Furthermore, phenolic compounds of active RTME were characterized by LC-MS/MS.
394 Eight compounds, two phenolic acids including quinic acid (RT3) and 5-caffeoylquinic acid
395 (RT4), five flavonoids including quercetin pentoside (RT6), quercetin glucoside (RT7),
396 quercetin-3-*O*-β-D-glucuronide (RT2), kaempferol-3-*O*-β-D-glucuronide (RT1) and
397 kaempferol rutinoside (RT8) and one coumarin including an unknown like coumarin derivative
398 (RT5) were detected in RTME (Table 5) [32,33]. RT3 and RT4 were determined as quinic acid
399 derivatives [36]. These compounds have previously been identified by Clifford et al. [34]. RT2,
400 RT6 and RT7 presented the same aglycon at m/z 301 which was identified as quercetin. RT6
401 showed a molecular ion peak at m/z 433$[M-H]^-$ which was 132 amu (most probably a pentose
402 sugar) higher than quercetin. Therefore, RT6 was identified as quercetin pentoside that has been
403 previously identified in *Rubus* species [33]. RT7 showed 162 amu (glucose) higher than
404 quercetin which was presented molecular ion peak at m/z 463 allowed us to identify RT7 as

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405 quercetin glucoside. The reason why RT2 was defined as quercetin glucuronide is that the
406 difference between the molecular weight of RT2 and quercetin is -176 amu (glucuronic acid).
407 It was also proven by the NMR spectrum that RT2 was exactly quercetin-3-*O*-β-D-glucuronide.
408 RT1 and RT8 presented the aglycon kaempferol at m/z 285 $[M-H]^-$. RT1 presented a molecular
409 ion peak at m/z 461 $[M-H]^-$ and then was fragmented to the aglycon kaempferol due to the loss
410 of -176 amu glucuronic acid. The fragmentation behavior enabled us to identify RT1 as
411 kaempferol glucuronide. It was also proven by the NMR spectrum that RT1 was precisely
412 kaempferol-3-*O*-β-D-glucuronide. RT8 showed a molecular ion peak at m/z 593 $[M-H]^-$ and
413 yielded the aglycon kaempferol due to the loss of -309 amu rutinose. Therefore, RT8 was
414 identified as kaempferol rutinoside. The identity of RT5 remained unknown in the present
415 study. Also, in a previous study, this compound was designated as unknown [33]. Due to the
416 presence of 161 and 133 ions, the compound appears to be coumarin-like. This makes us believe
417 that RT5 is an unidentified coumarin derivative.
418

419 **Table 5.** Characterization of phenolic compounds in the RTME
420

No	R_t min	$[M-H]^-$ *m/z*	MS^2	Identified as	References
RT3	3.2	191	173	Quinic acid	[32]
RT4	6.7	353	191, 173	5-caffeoylquinic acid	[32]
RT5	8.1	355	161, 133	Unknown like coumarin derivative	[33]
RT6	9.3	433	301, 283	Quercetin pentoside	[33]
RT7	10.5	463	301	Quercetin glucoside	[33]
RT2	10.8	477	301	Quercetin-3-O-β-D-glucuronide (Main compound)	[33]
RT1	12.1	461	285	Kaempferol-3-O-β-D-glucuronide	[33]
RT8	16.3	593	284	Kaempferol rutinoside	[33]

421

422 *3.6. In silico studies of major compounds isolated from RTME*

423 In an attempt to gain more insight into the molecular mechanisms underlying the anti-
424 inflammatory/antioxidant activity of RTME, we docked the bioactive constituents RT1 and
425 RT2 into the active-site cavities of COX-2 and inducible nitric oxide synthase (iNOS).
426 Redocking calculations based on the reference ligands celecoxib (for COX-2) and ethyl 4-[(4-

427 methylpyridin-2-yl)amino]piperidine-1-carboxylate (for iNOS) revealed that JAMDA was
428 successful in reproducing the co-crystallized poses of the ligands, with root-mean-square
429 deviations of 0.749513 Å (JAMDA score: -2.92094; Figure 5A) and 0.700049 Å (JAMDA
430 score: -2.56124; Figure 6A), respectively. Cross-docking calculations predicted that both RT1
431 and RT2 were able to occupy the active-site cavity of COX-2, with JAMDA scores of -2.28255
432 and -2.32281, respectively. They appeared to adopt similar binding poses here, and the
433 conformation of each flavonol glucuronide with respect to the active site was found to be
434 stabilized mainly by hydrogen-bonding interactions with the sugar moiety and various
435 hydrophobic π interactions with the two aromatic rings of the flavonol portion (Figures 5B, 5C).
436 Protein–ligand docking of RT1 and RT2 demonstrated that the flavonol glucuronides could be
437 housed well also in the active-site cavity of iNOS, with JAMDA scores of -2.31158 and -
438 2.19769, respectively (Figures 6B, 6C). They seemed to adopt almost identical binding poses
439 here, establishing electrostatic interactions and a rich network of hydrogen-bonding interactions
440 with the surrounding active-site residues though their sugar moieties. In addition, they were
441 able to engage in favorable interactions (hydrogen bonds and π–lone pair interactions) with the
442 enzyme's heme prosthetic group, exhibiting an inherent attribute of numerous potent iNOS
443 inhibitors.

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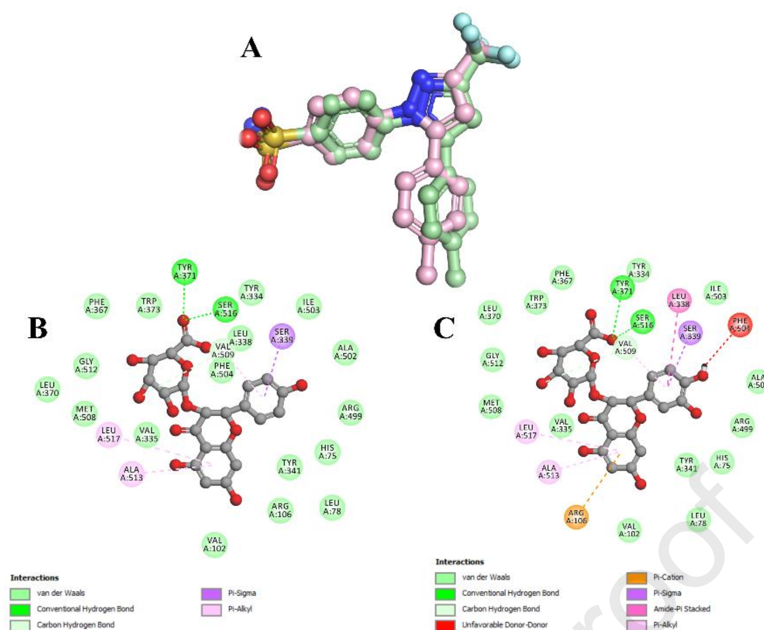

Figure 5. COX-2–ligand docking and interaction profiling. (**A**) The results of redocking calculations showing the superposed structures of the docked (light pink) and native (pale green) celecoxib molecule at the active site of COX-2. The image was rendered by using the PyMOL Molecular Graphics System, v1.8 (Schrödinger LLC, Portland, OR, USA). The results of cross-docking calculations showing the favorable non-covalent interactions that anchor the predicted binding poses of (**B**) RT1 and (**C**) RT2 to the active-site cleft of COX-2. The images were rendered by using Discovery Studio Visualizer, v16.1.0 (Dassault Systèmes BIOVIA Corp., San Diego, CA, USA).

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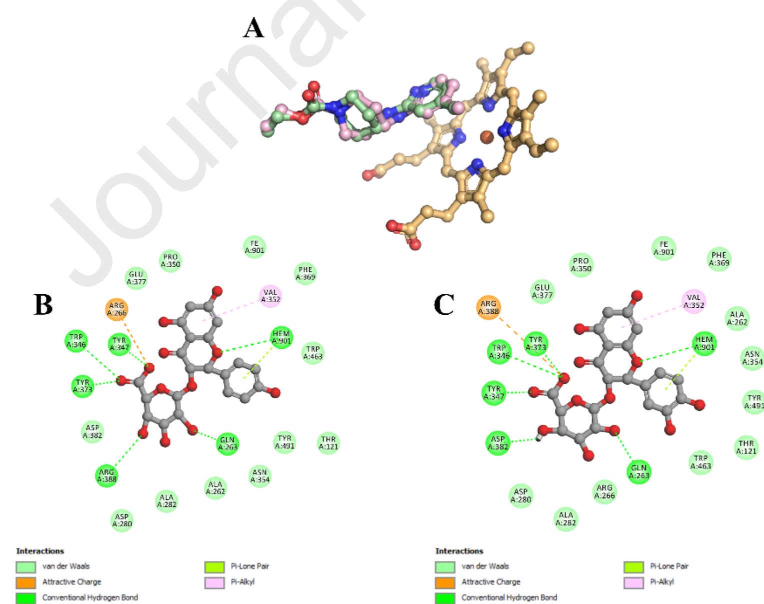

Figure 6. iNOS–ligand docking and interaction profiling. (**A**) The results of redocking calculations showing the superposed structures of the docked (light pink) and native (pale green) ethyl 4-[(4-methylpyridin-2-yl)amino]piperidine-1-carboxylate molecule relative to the heme prosthetic group (wheat) at the active site of iNOS. The image was rendered by using the PyMOL Molecular Graphics System, v1.8 (Schrödinger LLC, Portland, OR, USA). The results of cross-docking calculations showing the favorable non-covalent interactions that anchor the predicted binding poses of (**B**) RT1 and (**C**) RT2 to the active-site cleft of iNOS. The images were rendered by using Discovery Studio Visualizer, v16.1.0 (Dassault Systèmes BIOVIA Corp., San Diego, CA, USA).

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462 **4. Discussion**

463 Evaluation of the influence of *Rubus tereticaulis* ethanol extract on acetic acid-induced UC in
464 rats based on its traditional anti-inflammatory use, and *in vitro* anti-inflammatory and
465 antioxidant activity-guided isolation of active compounds from *Rubus tereticaulis* were
466 performed for the first time in this study.

467 Acetic acid-induced colitis, which shares many clinical similarities with human UC, is
468 considered an experimental model of intestinal inflammation [35]. Colonic tissue is known to
469 be sensitive to acetic acid. Such a situation causes an overproduction of the oxidative mediators
470 that play important roles in the pathophysiology of colitis. Pro-inflammatory mediators thought
471 to be responsible for IBD are ROS, hydrogen peroxide (H_2O_2), cytokines, and macrophage or
472 neutrophil recruitment to colonic mucosa [36]. Oxidative stress plays crucial roles in the
473 pathogenesis of UC [37], and it can be measured over several parameters such as nitric oxide
474 (NO), superoxide dismutase (SOD), GSH, MDA, and total antioxidant capacity (TAC) [38].

475 GSH serves as an important and essential cellular antioxidant that protects the cells from
476 the harmful effects of oxidative agents [39]. In agreement with our study, earlier studies have
477 also shown that GSH levels in the colon tissue tend to decrease in acetic acid-induced colitis
478 [39]. Here, we clearly showed that GSH levels significantly increased in the RTME treated
479 groups compared to the colitis group, highlighting the antioxidant potential of RTME. Zhang
480 et al. (2022), in a study investigating the protective effect of ethanol extract (RLEE) from
481 raspberry leaf on UVB-induced skin photo damage in the L929 fibroblast cell line, reported that
482 the extract increased the level of GSH in cells [40]. This result supports our current study and
483 shows that the leaves of *Rubus* species have an antioxidant activity.

484 Increased MDA levels in IBD are an important indicator of oxidative stress [41]. In a
485 study by Li et al., an increase in the concentrations of ROS and MDA (a measure of lipid
486 peroxidation) oxidative mediators was observed in the colon tissues of mice treated with DSS

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487 [42]. In the current study, MDA levels in colonic tissue were significantly increased in AA-
488 treated rats when compared to the control group. RTME treatment, however, was found to
489 significantly lower MDA levels in colonic tissues.

490 An important indicator reflecting the infiltration of neutrophils is the MPO activity in
491 the inflamed colon, which is normally increased in colitis groups compared to controls [43]. In
492 agreement with this interpretation, the highest MPO activity was observed in the colitis groups
493 in the present study. Treatment with RTME significantly reduced MPO activity in the colon
494 tissue accordingly.

495 Our macroscopic scoring data showed that AA-treated rats encountered excessive
496 damage to the colonic tissue, but the RTME and SS treatments exhibited significant
497 ameliorative effects on the condition. Also, our macroscopic results were concordant with our
498 biochemical results presented above.

499 In a study conducted by Stan *et al.*, it was suggested that the therapeutic effect of *Thuja*
500 *occidentalis* against UC may be due to its rich phenolic and flavonoid content [44].
501 Additionally, flavonoids have been reported to have beneficial effects in IBD [45]. In the
502 current study, the phytochemical content of RTME was investigated by the LC-MS/MS method,
503 and it was found that RTME is rich in phenolic acids and flavonoids, including quercetin-3-*O*-
504 β-D-glucuronide as major compound. This was verified by quantifying the total phenolic and
505 flavonoid content of RTME. It was also revealed that RTME displayed potent antioxidant and
506 anti-inflammatory activities *in vitro*. Therefore, it is tempting to speculate that RTME is
507 effective in protecting from colitis, possibly owing to its antioxidant and anti-inflammatory
508 capacity tightly associated with its high phenolic compound content.

509 In addition, kaempferol-3-*O*-β-D-glucuronide (RT1) and quercetin-3-*O*-β-D-
510 glucuronide (RT2) were isolated as major compounds from RTME by *in vitro* anti-
511 inflammatory and antioxidant activity-guided isolation. The presence of these compounds was

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512 confirmed by the LC-MS/MS analysis of RTME. RT1 [46,47,48,49] and RT2 [46,47,48,50,51]
513 have been reported to be found in different *Rubus* species such as *R. caesius, Rubus chingii, R.*
514 *coreanum, R. idaeus, R. ulmifolius* and *R. sachalinensis*. Also, Oszmiański et al. (2015) reported
515 that the most abundant flavonoid compounds in 26 different *Rubus* species were RT1 and RT2
516 [52]. These results were found to be in agreement with our current study. Additionally,
517 Tomczyk and Gudej (2005) stated that glycosylated derivatives were used as chemotaxonomic
518 markers in *Rubus* species. They reported that the formation of flavonol monoglycosides was
519 observed in these species, the glycosylation of these compounds at C-3 was the most frequent
520 substitution, and generally found as 3-*O*-glucosides, 3-*O*-galactosides, 3-*O*-glucuronides [53].
521 Therefore, the presence of RT1 and RT2 can be considered as a valuable chemophenetic marker
522 as one of the properties of *Rubus* species.

523 Of these two compounds, RT2 showed significant antioxidant activity against DPPH
524 and ABTS radicals with IC_{50} values of 7.71 µg/mL (16.12 µM) and 8.69 µg/mL (18.17 µM),
525 respectively. The activity of RT2 against the DPPH radical was investigated by different
526 researchers, and the corresponding IC_{50} values were reported to be 19.24 µg/mL, >523.438
527 µg/mL and 271.2 µM. On the other hand, RT2 was shown by other groups to exert anti-ABTS
528 radical activity, with an IC_{50} value of 115.9 µM [54-56]. The anti-LOX activities of RT1 and
529 RT2 was revealed for the first time in this study. RT1 and RT2 showed good anti-LOX activity
530 with an IC_{50} values of 49.14 µg/mL (106.28 µM) and 67.64 µg/mL (141.40 µM), respectively.
531 However, there are studies in the literature in which anti-inflammatory activities of these
532 compounds were evaluated by different methods. Egg albumin denaturation and proteinase
533 inhibitory activities of RT1 and RT2 were investigated, and it was reported that RT1 with IC_{50}
534 values of 25.4 and 63.2 µM and RT2 with IC_{50} values of 20.4 and 56.6 µM had anti-
535 inflammatory activity [57]. In another study, RT1 was reported to significantly inhibit multiple
536 pro-inflammatory factors such as IL-1β, NO, PGE2, and LTB4 in the LPS-induced RAW 264.7

537 cells and mouse models [58]. In a study conducted on mice, Fan et al. [59] deduced that RT2
538 showed its anti-inflammatory activity by significantly suppressing dimethyl benzene-induced
539 ear edema and AA-induced peritoneal permeability . In another study, Park et al. reported that
540 RT2 demonstrated its anti-inflammatory activity by suppressing the JNK and ERK signaling
541 pathways in the LPS-induced RAW 264.7 macrophage cells [60]. Our *in silico* findings, which
542 predict the inhibitory actions of RT1 and RT2 on COX-2 and iNOS activities, further support
543 the notion that these compounds are likely to possess considerable antioxidant/anti-
544 inflammatory activities in the body. When these pieces of evidence are viewed collectively, it
545 is safe to assume that the phenolic compounds in RTME, and RT2 in particular, are likely to be
546 responsible for the protective effects of the extract against colitis, owing to their strong
547 antioxidant and anti-inflammatory capacities.

548 **5. Conclusion**

549 Overall, RTME was found to have promising protective properties that can be of value in
550 alleviating colitis. It was also revealed that RT2 was the primary component responsible for the
551 observed bioactivity of RTME. Therefore, the results presented here both confirm the
552 traditional use of *R. tereticaulis* for inflammation-associated conditions and add to the relevant
553 scientific literature on the protective antioxidant and anti-inflammatory mechanisms of its
554 ethanolic leaf extract in an experimental model of colitis.

555

556 **Author contributions**

557 AS: conceptualization, methodology, software, investigation, formal analysis, writing original
558 draft preparation, writing—review & editing; DO: conceptualization, methodology, software,
559 investigation, formal analysis, writing original draft preparation, writing—review & editing;
560 KT: methodology, software, investigation, writing original draft preparation, writing—review
561 & editing; F.G: methodology, software, investigation, writing original draft preparation; AY:

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562 methodology, software, investigation, writing original draft preparation; BE: methodology,
563 software, investigation, writing original draft preparation; AD: methodology, writing—review
564 & editing; LB: methodology, investigation, writing—review & editing; GS: methodology,
565 investigation, writing—review & editing

566

567 **Conflict of interest disclosure**

568 The authors declare no conflict of interest.

569

570 **Data availability**

571 All the data generated or analyzed during this study are included in this published article.

572

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576

577 **Ethical approval**

578 All procedures for experimental protocols of the present study involving animals were
579 performed in accordance with the ethical standards of the institution of practice at which the
580 studies were conducted. This study was performed in line with the principles of the Declaration
581 of Helsinki. Approval was granted by the Ethics Committee of University Marmara
582 (12.10.2020/No:51.2020.mar).

583

584 **Appendix A. Supplementary data**

585 Supplementary data to this article can be found online at ……………………………………..

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Highlights

- First study on the phytochemical composition & anti-colitis activity of *Rubus tereticaulis* (RT).
- New report on anti-lipoxygenase activity of kaempferol-3-O-β-D-glucuronide and quercetin-3-O-β-D-glucuronide.
- RT significantly improved biochemical & macroscopic parameters in a rat model of colitis.
- Major components of RT were predicted to bind pro-oxidant/pro-inflammatory enzymes *in silico*.
- Overall, RT may serve as a promising agent in the treatment of ulcerative colitis.

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Author contributions

Ali Sen: conceptualization, methodology, software, investigation, formal analysis, writing original draft preparation, writing—review & editing; **Dilek Ozbeyli:** conceptualization, methodology, software, investigation, formal analysis, writing original draft preparation, writing—review & editing; **Kerem Terali:** methodology, software, investigation, writing original draft preparation, writing—review & editing; **Fatih Goger:** methodology, software, investigation, writing original draft preparation; **Aybeniz Yildirim:** methodology, software, investigation, writing original draft preparation; **Busra Ertas:** methodology, software, investigation, writing original draft preparation; **Ahmet Dogan:** methodology, writing—review & editing; **Leyla Bitis:** methodology, investigation, writing—review & editing; **Goksel Sener:** methodology, investigation, writing—review & editing

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Declaration of interests

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

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

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