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Research paper

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

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ABSTRACT

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

1. Introduction

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

Oxidative stress and inflammation form the basis of the pathogenesis of many diseases. Free radicals produced by the physical and biochemical processes that occur in the human body damage various biomolecules that are important for the body, thereby causing various diseases [2]. Inflammation is an indeterminate physiological response of the body to damage caused by endogenous or exogenous agents, and is an inevitable limited response of the body to spontaneous exposure when the tissue is exposed to chemical, mechanical or biological damage. Therefore, inflammation acts as one of the defense barriers of the

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organism. Keeping inflammation under control plays an important role in the treatment of chronic inflammation-related diseases such as arthritis, osteoarthritis, sclerosis, arteriosclerosis, Alzheimer's, diabetes, insulin-resistance, obesity, allergies, asthma, chronic bronchitis, cancer, tuberculosis, retinitis, psoriasis, lung fibrosis, and chronic gastritis [3].

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

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

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

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

2. Materials and methods

2.1. Plant material

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

2.2. Extraction

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

2.3. Determination of in vitro anti-inflammatory activity

The anti-LOX activity was evaluated as described by Phosrithong and Nuchtavorn [2]. 500 μL of extracts, isolated compounds and standard indomethacin were added to 250 μL of sodium borate buffer solution (0.1 M, pH 9) followed by addition of 250 μL of type V soybean LOX solution in buffer (pH 9, 20.000 U/mL). The mixture was preincubated at 25 °C for 5 min. Then, 1000 μL of 0.6 mM linoleic acid solution was added, mixed well and the change in absorbance at 234 nm was recorded for 6 min. The percent inhibition was calculated from the following equation:

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

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

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

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

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

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

2.5. Determination of 2.2′-azinobis (3-ethylbenzothiazolin-6-sulphonic acid (ABTS) radical-scavenging activity

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

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

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

2.6. Animals and experimental design

Wistar albino rats (250–300 g) were supplied by the Marmara University (MU) Application and Research Center for Experimental Animals (DEHAMER). Rats were housed in an air-conditioned room with 12:12 light:dark cycles, where the temperature (22 ± 2 °C) and relative humidity (65–70%) were kept constant. All experimental protocols were approved by the MU Animal Care and Use Committee (Protocol number: 51.2020. mar). The rats were randomly divided into five groups, with six rats in each group: control group; 300 mg/kg *Rubus* group; colitis (AA)+ physiological saline group; colitis+300 mg/kg *Rubus* group; and colitis+100 mg/kg sulfasalazine (SS) group. All sets of treatment regimens were given by oral gavage and administered as a single dose daily. Treatment was started following colitis induction and continued for 3 consecutive days.

2.7. Induction of ulcerative colitis

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

2.8. Assessment of colitis severity

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

2.9. Measurement of tissue MDA and GSH levels

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

2.10. Measurement of tissue MPO activity

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

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

2.11. Isolation of active compounds from RTME

In *in vitro* bioactivity experiments, RTMEA showed the best antioxidant and anti-LOX activity among all fractions of RTME. Therefore, RTMEA was chosen for further isolation experiments. RTMEA (2.0853 g) was fractionated by CC on Sephadex, using CH_3OH to yield forty-two fractions. Fractions exhibiting similar TLC profiles (TLC silica gel 60 F_{254} plates were developed using 8:1:1 ethyl acetate:formic acid:water as eluent) were combined to give four sub-fractions (F1–F6: 0.4996 g; F7–F15: 0.5599 g; F16–F27: 0.2827 g; F28–F42: 0,2294 g). DPPH and anti-inflammatory activity tests were performed utilizing these fractions. We continued the isolation with F7–F15, having the highest activity and the most intense compound content on TLC among all fractions, F7–F15 (0.5537 g) was repeatedly subjected to size-exclusion chromatography on a Sephadex LH-20 column, eluted with CH_3OH and then combined sub-fractions was re-chromatographed by preparative TLC with ethyl acetate:formic acid:water (8:1:1) to give RT1 (30.3 mg) and RT2 (23.4 mg) (Fig. 1)

2.12. LC-MS/MS analysis of RTME

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

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

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

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

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

2.15. Protein–ligand docking and interaction profiling

The SMILES strings of RT1 and RT2 were translated into energy-minimized 3D structures with appropriate topologies and parameters by using the myPresto program suite (available at https://demo1.biomodeling.co.jp/). The 3D structures of COX-2 (PDB ID: 3LN1; [25])

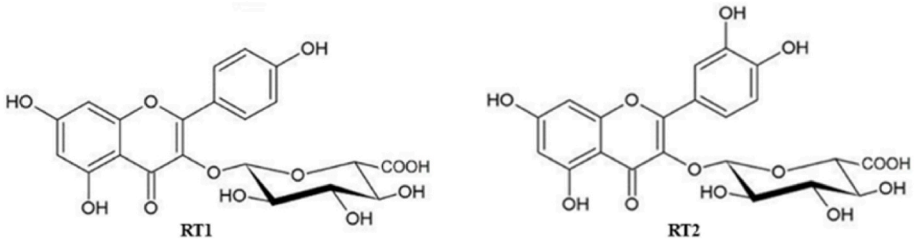

Fig. 1. Chemical structures of major compounds isolated from *R. tereticaulis*.

and iNOS (PDB ID: 3E7G; [26]) in complex with selective small-molecule inhibitors were retrieved from the RCSB Protein Data Bank ([27]; available at https://www.rcsb.org/). Protein–ligand docking was achieved with high precision by using JAMDA (available at https://proteins.plus/) that combines the TrixX docking algorithm [28,29] with the JAMDA scoring function [30]. The protein was prepared by keeping all heteroatoms (except those of the reference ligand and water molecules within the binding site), and the most likely protonation states and hydrogen coordinates were assigned to the protein through Protoss optimization. The binding site was defined by the reference ligand, with a site radius of 6.5 Å. The docking poses generated were prioritized based on their JAMDA scores. Favorable non-covalent interactions between the proteins and ligands were computed by using Discovery Studio v16.1.0 (Dassault Systèmes BIOVIA Corp., USA).

2.16. Statistical analysis

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

3. Results

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

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

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

The results of the *in vitro* tests showed that RTME had strong antioxidant activity, with IC_{50} values of 18.31 μg/mL and 13.91 μg/mL against DPPH and ABTS radicals, respectively. RTME exhibited significant anti-inflammatory activity by inhibiting the development of LOX enzyme activity by 91.15% at a concentration of 156 μg/mL (Table 2). Also, the amounts of total phenolics and flavonoids of RTME were found to be 35.55 and 6.94 mg/g, respectively (Table 3).

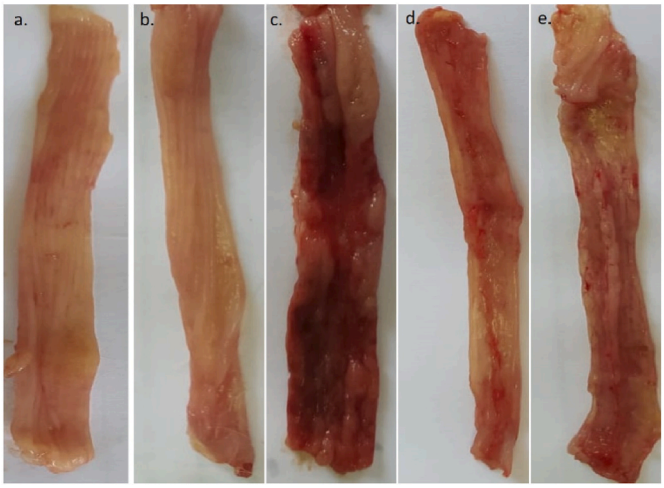

Fig. 2. Macroscopic appearance of colonic tissues of **a)** control group, **b)** 300 mg/kg *Rubus* group, **c)** colitis (AA)+physiological saline group, **d)** colitis+300 mg/kg *Rubus* group, **e)** colitis+100 mg/kg sulfasalazine (SS) group.

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

RTME demonstrated significant bioactivity in both *in vitro* and *in vivo* assays. Therefore, in an attempt to reveal the compounds that were responsible for this behavior, we decided to perform bioassay-directed fractionation and subsequent isolation. Firstly, RTMH, RTMC, RTMEA and RTMAE fractions were obtained by liquid–liquid extraction from RTME. Then, *in vitro* antioxidant and anti-inflammatory activities of these fractions as well as total phenol and flavonoid contents were evaluated. RTMEA with IC_{50} values 12.34 and 9.00 μg/mL against ABTS and DPPH radicals showed significant antioxidant activity when compared to other fractions ($p < 0.05$) (Table 2). Also, this fraction exhibited the highest anti-inflammatory activity against 5-lipoxygenase at concentration of 156 μg/mL ($p < 0.05$) (Table 2). In addition, RTMEA had the most significant ($p < 0.05$) amount of phenolic (63.30 mg/g) and flavonoid (12.76 mg/g) compared to the other fractions (Table 3). Based on these results, priority was given to the ethyl acetate fraction of RTME (RTMEA) for active compound isolation. Of the obtained 4 subfractions (F1–F6; F7–F15; F16–F27; F28–F42), the isolation process was continued with F7–F15, which showed the best activity. [DPPH radical and LOX inhibition rate at a concentration of 50 μg/mL: 50.50 and 19.77% (F1–F6), 82.86 and 96.05% (F7–F15), 82.03 and 72.01% (F16–F27), 82.03 and 76.02% (F28–F42), respectively]. Two flavonol glucuronides, kaempferol-3-*O*-*β*-D-glucuronide (RT1, 30.3 mg) and quercetin-3-*O*-*β*-D-glucuronide (RT2, 23.4 mg), were isolated as major compounds from the active F7–F15 sub-fraction of RTMEA (Fig. 1). All isolated compounds were analyzed by spectroscopic methods (UV, ^{1}H NMR, and ^{13}C NMR-APT), and the resulting data were compared with those reported in the literature. (Detailed spectral data is included in supporting information.)

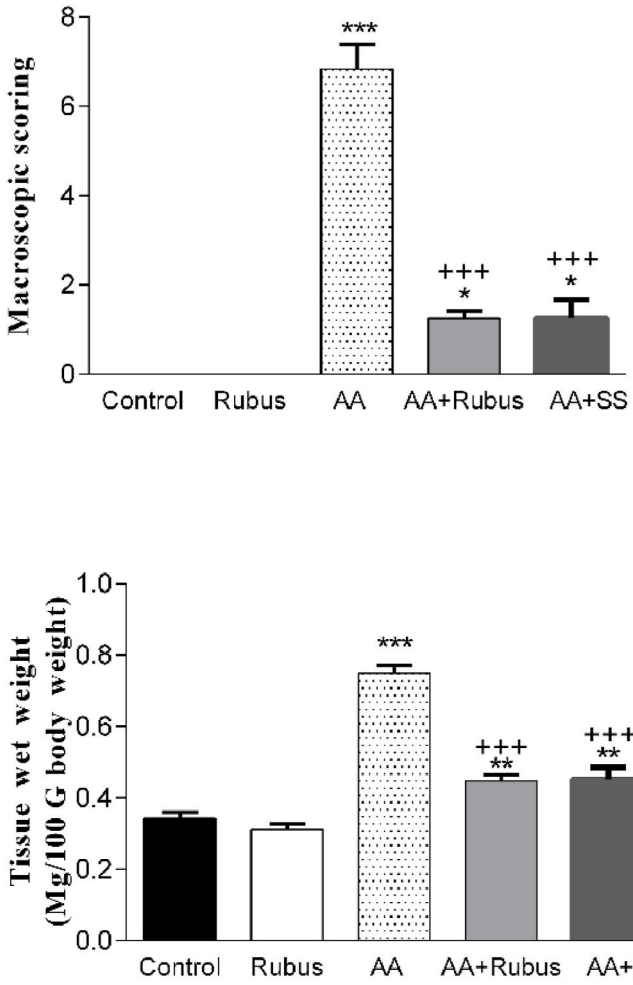

Fig. 3. Macroscopic lesion scores and wet weight of colonic tissues of control group, 300 mg/kg *Rubus* group, colitis (AA)+physiological saline group, colitis+300 mg/kg *Rubus* group, colitis+100 mg/kg sulfasalazine (SS) group. Values are represented as mean ± sem. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p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

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

The *in vitro* antioxidant activities of the compounds were evaluated according to the classification criteria described by Ervina et al. [31]. Normal classification of a compound's antioxidant activity generally follows the followings criteria: a very powerful antioxidant (IC_{50}: <50 μg/mL); a strong antioxidant (IC_{50}: 50–100 μg/mL); an intermediate antioxidant (IC_{50}: 101–150 μg/mL); and a weak antioxidant (IC_{50}: 151–200 μg/mL) [36]. When the antioxidant activities of RT1 and RT2, which are the major compounds isolated from active ethyl acetate fraction of RTME, were evaluated, especially RT2 was found to exhibit significant antioxidant activity against DPPH and ABTS radicals with IC_{50} values of 7.71 and 8.69 μg/mL, respectively. Furthermore, RT1 with an IC_{50} of 49.14 μg/mL showed better anti-LOX activity than RT2 (IC_{50}: 67.64 μg/mL) (Table 4).

3.5. LC-MS/MS analysis of RTME

Furthermore, phenolic compounds of active RTME were characterized by LC-MS/MS. Eight compounds, two phenolic acids including quinic acid (RT3) and 5-caffeoylquinic acid (RT4), five flavonoids including quercetin pentoside (RT6), quercetin glucoside (RT7), quercetin-3-*O*-β-D-glucuronide (RT2), kaempferol-3-*O*-β-D-glucuronide (RT1)

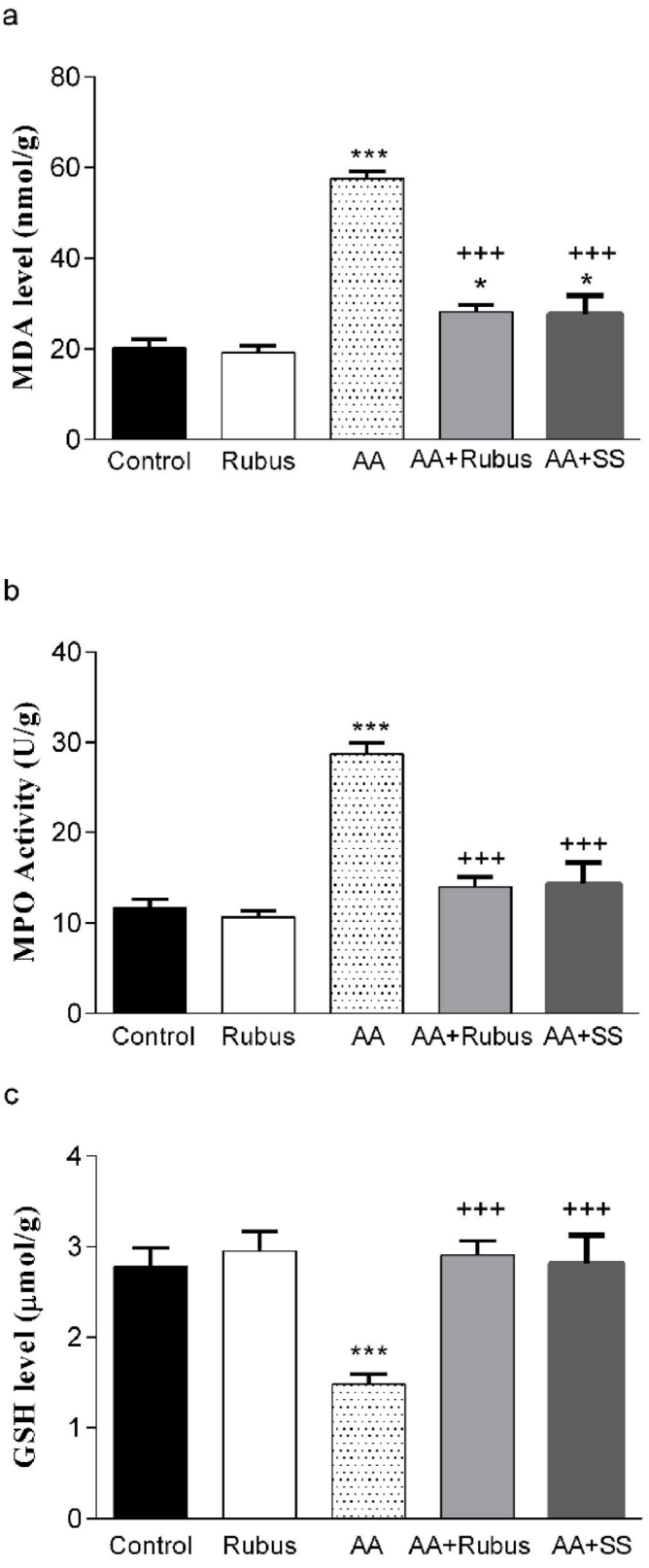

Fig. 4. Malondialdehyde (MDA) levels, myeloperoxidase (MPO) activities and glutathione (GSH) levels in the colonic tissues of control group, 300 mg/kg *Rubus* group, colitis (AA)+physiological saline group, colitis+300 mg/kg *Rubus* group, colitis+100 mg/kg sulfasalazine (SS) group. Values are represented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

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

Extracts[a]	Yields	ABTS activity IC_{50} (μgmL^{-1})[b]	DPPH activity IC_{50} (μgmL^{-1})[b]	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]

***Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

[a] The ethanol extract and its n-hexane, chloroform, ethyl acetate and aqueous ethanol fractions obtained by maceration and liquid–liquid extraction were coded as RTME, RTMH, RTMC, RTMEA and RTMAE, respectively. The yields of RTME was calculated from the powdered dry plant. The yields of the remaining extracts were calculated from dried RTME.

[b] IC_{50}: Values corresponding to the concentration of extract required to scavenge/inhibit 50% of radicals/enzyme present in the reaction mixture.

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

Extracts[a]	TPC[b] (mg GAE/g extract)	TFC[c] (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]

****Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

[a] The ethanol extract and its n-hexane, chloroform, ethyl acetate and aqueous ethanol fractions obtained by maceration were coded as RTME, RTMH, RTMC, RTMEA and RTMAE, respectively.

[b] Total phenolic content (TPC) was expressed as gallic acid equivalent (GAE).

[c] Total flavonoid content (TFC) was expressed as catechin equivalent (CE).

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

Compounds[a]	DPPH activity	ABTS activity	Anti-inflammatory activity
	IC_{50} (μgmL^{-1})[b]		
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]

[a] Kaempferol-3-*O*-*β*-D-glucuronide and quercetin-3-*O*-*β*-D-glucuronide were coded as RT1 and RT2, respectively.

[b] Each value in the table is represented as mean ± SEM (n = 3). Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

and kaempferol rutinoside (RT8) and one coumarin including an unknown like coumarin derivative (RT5) were detected in RTME (Table 5) [32,33]. RT3 and RT4 were determined as quinic acid derivatives [36]. These compounds have previously been identified by Clifford et al. [34]. RT2, RT6 and RT7 presented the same aglycon at *m/z* 301 which was identified as quercetin. RT6 showed a molecular ion peak at *m/z* 433[M − H]$^-$ which was 132 amu (most probably a pentose sugar) higher than quercetin. Therefore, RT6 was identified as quercetin pentoside that has been previously identified in *Rubus* species [33]. RT7 showed 162 amu (glucose) higher than quercetin which was presented molecular ion peak at *m/z* 463 allowed us to identify RT7 as quercetin glucoside. The reason why RT2 was defined as quercetin glucuronide is that the difference between the molecular weight of RT2 and quercetin is −176 amu (glucuronic acid). It was also proven by the NMR spectrum that RT2 was exactly quercetin-3-*O*-β-D-glucuronide. RT1 and RT8 presented the aglycon kaempferol at *m/z* 285 [M − H]$^-$. RT1 presented a molecular ion peak at *m/z* 461[M − H]$^-$ and then was fragmented to the aglycon kaempferol due to the loss of −176 amu glucuronic acid. The fragmentation behavior enabled us to identify RT1 as kaempferol glucuronide. It was also proven by the NMR spectrum that RT1 was precisely kaempferol-3-*O*-β-D-glucuronide. RT8 showed a molecular ion peak at *m/z* 593 [M − H]$^-$ and yielded the aglycon kaempferol due to the loss of −309 amu rutinose. Therefore, RT8 was identified as kaempferol rutinoside. The identity of RT5 remained unknown in the present study. Also, in a previous study, this compound was designated as unknown [33]. Due to the presence of 161 and 133 ions, the compound appears to be coumarin-like. This makes us believe that RT5 is an unidentified coumarin derivative.

Table 5
Characterization of phenolic compounds in the RTME.

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]

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

In an attempt to gain more insight into the molecular mechanisms underlying the anti-inflammatory/antioxidant activity of RTME, we docked the bioactive constituents RT1 and RT2 into the active-site cavities of COX-2 and inducible nitric oxide synthase (iNOS). Redocking calculations based on the reference ligands celecoxib (for COX-2) and ethyl 4-[(4-methylpyridin-2-yl)amino]piperidine-1-carboxylate (for iNOS) revealed that JAMDA was successful in reproducing the co-crystallized poses of the ligands, with root-mean-square deviations of 0.749513 Å (JAMDA score: −2.92094; Fig. 5A) and 0.700049 Å (JAMDA score: −2.56124; Fig. 6A), respectively. Cross-docking calculations predicted that both RT1 and RT2 were able to occupy the active-site cavity of COX-2, with JAMDA scores of −2.28255 and −2.32281, respectively. They appeared to adopt similar binding poses here, and the conformation of each flavonol glucuronide with respect to the active site was found to be stabilized mainly by hydrogen-bonding interactions with the sugar moiety and various hydrophobic π interactions with the two aromatic rings of the flavonol portion (Fig. 5B and C). Protein–ligand docking of RT1 and RT2 demonstrated that the flavonol glucuronides could be housed well also in the active-site cavity of iNOS, with JAMDA scores of −2.31158 and −2.19769, respectively (Fig. 6B and C). They seemed to adopt almost identical binding poses here, establishing electrostatic interactions and a rich network of hydrogen-bonding interactions with the surrounding active-site residues though their sugar moieties. In addition, they were able to engage in favorable interactions (hydrogen bonds and π–lone pair interactions) with the

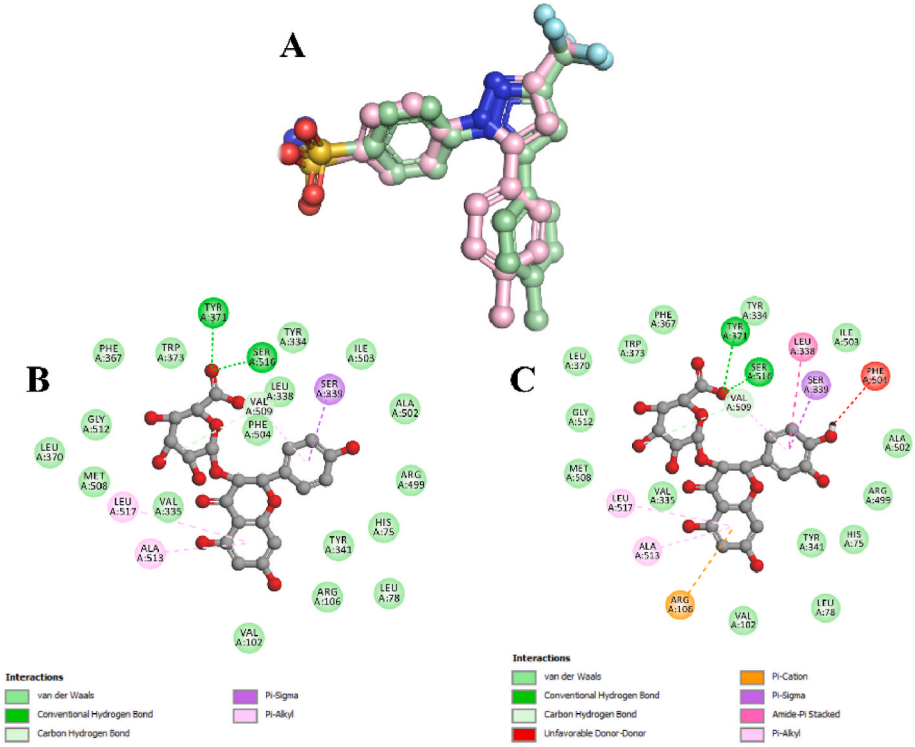

Fig. 5. COX-2–ligand docking and interaction profiling. (**A**) The results of redocking calculations showing the superposed structures of the docked (light pink) and native (pale green) celecoxib molecule at the active site of COX-2. The image was rendered by using the PyMOL Molecular Graphics System, 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).

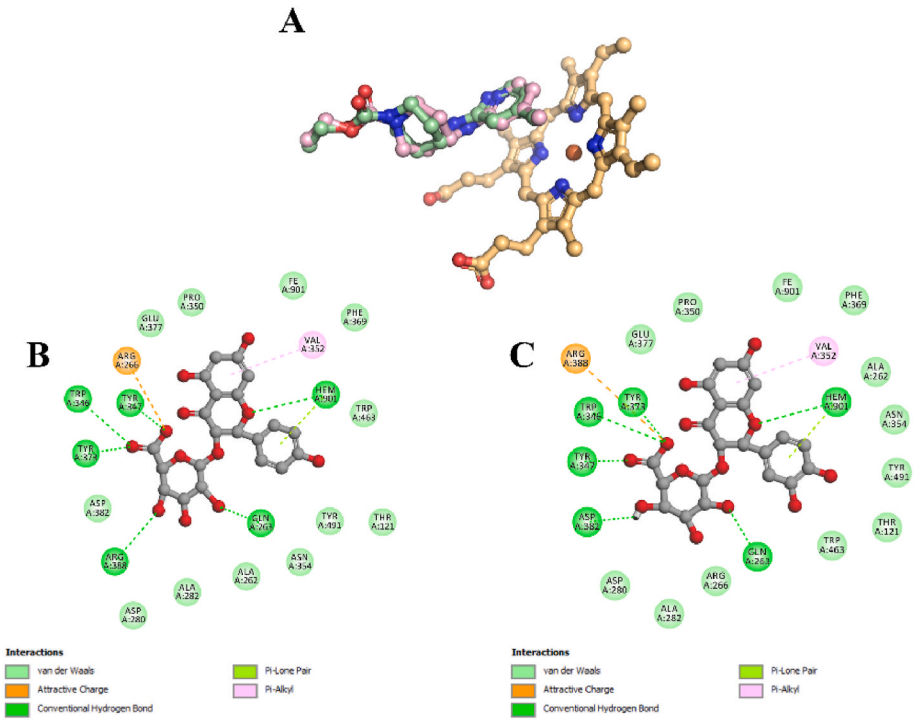

Fig. 6. iNOS–ligand docking and interaction profiling. (**A**) The results of redocking calculations showing the superposed structures of the docked (light pink) and native (pale green) ethyl 4-[(4-methylpyridin-2-yl)amino]piperidine-1-carboxylate molecule relative to the heme prosthetic group (wheat) 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).

enzyme's heme prosthetic group, exhibiting an inherent attribute of numerous potent iNOS inhibitors.

4. Discussion

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

Acetic acid-induced colitis, which shares many clinical similarities with human UC, is considered an experimental model of intestinal inflammation [35]. Colonic tissue is known to be sensitive to acetic acid. Such a situation causes an overproduction of the oxidative mediators that play important roles in the pathophysiology of colitis. Pro-inflammatory mediators thought to be responsible for IBD are ROS,

hydrogen peroxide (H_2O_2), cytokines, and macrophage or neutrophil recruitment to colonic mucosa [36]. Oxidative stress plays crucial roles in the pathogenesis of UC [37], and it can be measured over several parameters such as nitric oxide (NO), superoxide dismutase (SOD), GSH, MDA, and total antioxidant capacity (TAC) [38].

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

Increased MDA levels in IBD are an important indicator of oxidative stress [41]. In a study by Li et al., an increase in the concentrations of ROS and MDA (a measure of lipid peroxidation) oxidative mediators was observed in the colon tissues of mice treated with DSS [42]. In the current study, MDA levels in colonic tissue were significantly increased in AA-treated rats when compared to the control group. RTME treatment, however, was found to significantly lower MDA levels in colonic tissues.

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

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

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

In addition, kaempferol-3-*O*-β-D-glucuronide (RT1) and quercetin-3-*O*-β-D-glucuronide (RT2) were isolated as major compounds from RTME by *in vitro* anti-inflammatory and antioxidant activity-guided isolation. The presence of these compounds was confirmed by the LC-MS/MS analysis of RTME. RT1 [46–49] and RT2 [46–48,50,51] have been reported to be found in different *Rubus* species such as *R. caesius, Rubus chingii, R. coreanum, R. idaeus, R. ulmifolius* and *R. sachalinensis*. Also, Oszmiański et al. (2015) reported that the most abundant flavonoid compounds in 26 different *Rubus* species were RT1 and RT2 [52]. These results were found to be in agreement with our current study. Additionally, Tomczyk and Gudej (2005) stated that glycosylated derivatives were used as chemotaxonomic markers in *Rubus* species. They reported that the formation of flavonol monoglycosides was observed in these species, the glycosylation of these compounds at C-3 was the most frequent substitution, and generally found as 3-*O*-glucosides, 3-*O*-galactosides, 3-*O*-glucuronides [53]. Therefore, the presence of RT1 and RT2 can be considered as a valuable chemophenetic marker as one of the properties of *Rubus* species.

Of these two compounds, RT2 showed significant antioxidant activity against DPPH and ABTS radicals with IC_{50} values of 7.71 μg/mL (16.12 μM) and 8.69 μg/mL (18.17 μM), respectively. The activity of RT2 against the DPPH radical was investigated by different researchers, and the corresponding IC_{50} values were reported to be 19.24 μg/mL, >523.438 μg/mL and 271.2 μM. On the other hand, RT2 was shown by other groups to exert anti-ABTS radical activity, with an IC_{50} value of 115.9 μM [54–56]. The anti-LOX activities of RT1 and RT2 was revealed for the first time in this study. RT1 and RT2 showed good anti-LOX activity with an IC_{50} values of 49.14 μg/mL (106.28 μM) and 67.64 μg/mL (141.40 μM), respectively. However, there are studies in the literature in which anti-inflammatory activities of these compounds were evaluated by different methods. Egg albumin denaturation and proteinase inhibitory activities of RT1 and RT2 were investigated, and it was reported that RT1 with IC_{50} values of 25.4 and 63.2 μM and RT2 with IC_{50} values of 20.4 and 56.6 μM had anti-inflammatory activity [57]. In another study, RT1 was reported to significantly inhibit multiple pro-inflammatory factors such as IL-1β, NO, PGE2, and LTB4 in the LPS-induced RAW 264.7 cells and mouse models [58]. In a study conducted on mice, Fan et al. [59] deduced that RT2 showed its anti-inflammatory activity by significantly suppressing dimethyl benzene-induced ear edema and AA-induced peritoneal permeability. In another study, Park et al. reported that RT2 demonstrated its anti-inflammatory activity by suppressing the JNK and ERK signaling pathways in the LPS-induced RAW 264.7 macrophage cells [60]. Our *in silico* findings, which predict the inhibitory actions of RT1 and RT2 on COX-2 and iNOS activities, further support the notion that these compounds are likely to possess considerable antioxidant/anti-inflammatory activities in the body. When these pieces of evidence are viewed collectively, it is safe to assume that the phenolic compounds in RTME, and RT2 in particular, are likely to be responsible for the protective effects of the extract against colitis, owing to their strong antioxidant and anti-inflammatory capacities.

5. Conclusion

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

Author contributions

AS: conceptualization, methodology, software, investigation, formal analysis, writing original draft preparation, writing—review & editing; DO: conceptualization, methodology, software, investigation, formal analysis, writing original draft preparation, writing—review & editing; KT: methodology, software, investigation, writing original draft preparation, writing—review & editing; F.G: methodology, software, investigation, writing original draft preparation; AY: methodology, software, investigation, writing original draft preparation; BE: methodology, software, investigation, writing original draft preparation; AD: methodology, writing—review & editing; LB: methodology, investigation, writing—review & editing; GS: methodology, investigation, writing—review & editing.

Ethical approval

All procedures for experimental protocols of the present study involving animals were performed in accordance with the ethical standards of the institution of practice at which the studies were conducted.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University Marmara (October 12, 2020/No:51.2020.mar).

Declaration of competing interest

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2022.110263.

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