Jasonia glutinosa (L.) DC., a traditional herbal medicine, reduces inflammation, oxidative stress and protects the intestinal barrier in a murine model of colitis

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Abstract

Jasonia glutinosa (L.) DC., known as rock tea (RT), is traditionally used in Spain as a digestive due to its beneficial properties in bowel disorders. The pharmacological nature of these properties has not been established yet. The aim of this work was to evaluate the therapeutic utility of RT in experimental colitis and to identify chemical constituents with anti-inflammatory and/or anti-oxidative properties.

RT extract was prepared with ethanol in a Soxhlet apparatus and analysed by HPLC-DAD. Superoxide radical scavenging properties, xanthine oxidase and lipoxygenase (5-LOX) inhibitory activity, and capability to lower nitric oxide (NO) and tumour necrosis factor α (TNF-α) levels were measured in cell-free and cell-based assays. In the 2.5%-dextran-sodium sulphate (DSS) injury-repair model of ulcerative colitis (UC), mice were daily treated with sulfasalazine (SSZ, as reference drug, 100 mg/kg bw), RT (5, 25 and 50 mg/kg bw, p.o.), or vehicle over 20-days. Colitis was scored daily. Colon samples were macroscopically and histopathologically examined. Protein levels of myeloperoxidase (MPO), interleukins 6, and 10 (IL-6, IL-10), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) were studied as markers of oxidative stress and inflammatory activity. The integrity of the apical epithelial layer was assessed by immunofluorescence staining of zonula occludens-1 (ZO-1). Finally, intestinal contractility was also evaluated by isometric myography.

Fifteen phenolic compounds and three pigments were identified and quantified, of which caffeoylquinic acids, and the flavonoid, quercetin-3-O-galactoside, were the most abundant. RT extract significantly scavenged superoxide radicals, inhibited 5-LOX activity, and lowered NO and TNF-α levels. DSS-treated mice receiving RT scored clinically lower than controls during the first 3-days of DSS-treatment and during the recovery period. SSZ was less effective than RT. Anatomical and histological examination of colon samples revealed that RT significantly prevented shortening and thickening, and lowered the macroscopic damage score. RT also significantly prevented the increase of MPO activity, IL-6 levels, iNOS and COX-2 expression, the loss of ZO-1 apical expression, and normalized contractility disturbances.

In conclusion, daily administration of RT showed therapeutic properties in the DSS-model of UC. The benefits of RT can likely be attributed to its anti-inflammatory and antioxidant phenolic and flavonoid constituents.

Keywords list: inflammation; murine; polyphenols; rock tea; ulcerative colitis; ethnopharmacology

List of abbreviations

ACh: acetylcholine, AUC: area under the curve, COX-2: cyclooxygenase-2, CV: coefficient of variation, DAI: disease activity index, DMSO: dimethyl sulfoxide, DSS: dextran sodium sulphate, HE: haematoxylin-eosin, HPLC-DAD: high pressure liquid chromatography coupled to a diode-array detector, iNOS: inducible nitric oxide synthase, IBD: inflammatory bowel disease, IL-6: interleukin 6; IL-10: interleukin 10, LOD: limit of detection, LOQ: limit of quantification, 5-LOX: 5-lipoxygenase, LPS: lipopolysaccharide, MPO: myeloperoxidase, NADH/PMS: β-nicotinamide adenine dinucleotide/phenazine methosulfate, NBT: nitrotetrazolium blue chloride, NO: nitric oxide, $O_2^{\bullet-}$: superoxide radical, RT: rock tea, SSZ: sulfasalazine, TNF-α: tumour necrosis factor-alpha, UC: ulcerative colitis, X/XO: xanthine/xanthine oxidase, ZO-1: zonula occludens-1.

1. Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD), which is characterized by inflammation of the intestinal wall and ulcers in colon and rectum. The prevalence and incidence of UC is increasing over the years (Molodecky et al. 2012). The classical clinical features are weight loss, bloody stool, abdominal pain and intestinal motility disorders as diarrhoea or tenesmus. The aetiology is not fully understood, although genetic and environmental factors have been proposed (Baumgart and Carding 2007; Baumgart and Sandborn 2007; Danese and Fiocchi 2011; Low et al. 2013). The main histopathological feature of UC is the damage of the intestinal mucosa (epithelial layer), resulting in a loss of the defensive epithelial barrier, leading to increased permeability and an inflammatory response (Silva et al. 2016). Under inflammatory conditions, the morphology and structure of interstitial cells of Cajal can be affected, producing changes in the gastrointestinal motility (Dai et al. 2017; Won et al. 2006). In addition, dysmotility can increase the overgrowth of bacteria, the release of toxic products as well as their translocation through the barrier of the intestinal mucosa, increasing the severity of the condition.

Currently, drug therapies for UC based on salicylates and corticosteroids are not satisfactory, can be costly and may have important side effects. Therefore, there is a clinical need for new therapeutic strategies that may also derive from traditional herbal medicine (Low et al. 2013; Zheng et al. 2017).

Jasonia glutinosa (L.) DC. (Compositae), popularly known as rock tea (RT), is a medicinal plant species of the Iberian Peninsula and southern France. Infusions and decoctions are described as digestives and are used as substitutes for coffee or tea after meals due to the fact that this medicinal plant has been reported to relieve common gastrointestinal disturbances, such as diarrhoea or abdominal pain (Alarcón et al. 2015; Pardo Santayana and Morales 2004). Other traditional uses include the treatment of appendicitis, respiratory diseases and as analgesic, antiemetic or antidepressant medicinal plant (Akerreta et al. 2007; Pardo Santayana and Morales 2004).

Although certain studies with *J. glutinosa* have shown antioxidant (López et al. 2008), anti-inflammatory (Bermejo et al. 2002), antimicrobial (Villaescusa-Castillo et al. 2000; Villaescusa et al. 1996), antihypertensive (Valero et al. 2015) and spasmolytic properties (Castro et al. 2016), there is still a considerable lack of *in-vivo* studies supporting these traditional uses. In fact, studies involving the effects of this medicinal plant on the clinical symptoms of intestinal inflammation have never been performed or published. To the best of our knowledge, most of the existing studies concerning the chemical composition of *J. glutinosa* focus on its volatile composition (Valero et al. 2013). Although previous works report the presence of some flavonoids (Rubio et al. 1995; Villaescusa et al. 1995), there is a gap in the biologically-relevant metabolites knowledge of this plant. Therefore, we here aim to examine whether oral administration of RT extract is beneficial in the standard dextran sodium sulphate (DSS)-model of colitis. So, we evaluated the efficacy of RT to ameliorate the clinical progression of colitis, the microscopic and macroscopic colonic damage, loss of intestinal barrier and motility disturbances as well as its antioxidant and anti-inflammatory properties. Phenolic and carotenoid profiles were also determined by liquid chromatography (HPLC-DAD), in an attempt to identify therapeutically active constituents.

2. Materials and Methods

2.1. Standards and reagents

Standards of ascorbic acid, linoleic acid, caffeic acid, quercetin, lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, kaempferol and isorhamnetin were from Extrasynthèse (Genay, France). 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 1,3-di-*O*-caffeoylquinic acid, isoferulic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 1,5-di-*O*-caffeoylquinic acid, and 4,5-dicaffeoylquinic acid were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). Pheophytin *a* was from LGC Standards (Irvine, CA, US).

Potassium dihydrogen phosphate (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), nitrotetrazolium blue chloride (NBT), lipoxygenase (5-LOX) from *Glycine max* (L.) Merr. (type V-S; EC 1.13.11.12), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), *tert*-butyl methyl ether (MTBE), gallic acid, xanthine, NBT, xanthine oxidase, DPPH, galantamine, ATCI, DTNB, Tris, vanillic acid, 4-aminoantipyrine, horseradish peroxidase, LPS (lipopolysaccharide from *Escherichia coli* serotype 011:B4), RPMI Medium 1640-GlutaMAX^{TM}-I, trypsin, penicillin G, streptomycin, dimethyl sulfoxide (DMSO), sodium nitrite ($NaNO_2$), compounds of Griess reactive and acetylcholine were purchased from Sigma-Aldrich. HPLC grade methanol was obtained from Merck (Darmstadt, Germany) and formic acid was purchased from BDH Prolab (Dublin, Ireland). Dextran sodium sulphate (DSS) was purchased from Panreac.

Water was deionized in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Plant material and extraction

Collection of the plant and preparation of the ethanolic extract were performed as described in Valero *et al*., (Valero et al. 2015) following The United Nations Convention on Biological Diversity. Samples were authenticated and a voucher specimen (ref. 001-2012) has been deposited at the Department of Pharmacy of Universidad San Jorge. Solvent (ethanol) was removed using a rotary evaporator under vacuum and samples were kept at – 20 ºC until bioassays.

2.3. Phytochemical analyses

2.3.1. HPLC-DAD analysis

The dried ethanolic extract of *J. glutinosa* was reconstituted in high quality methanol and filtered with a membrane (pore size:0.45 μm) before chromatographic analysis.

2.3.1.1. Phenolic compounds determination

20 μL of re-dissolved and filtered extract was analysed using a Gilson HPLC-DAD unit with a Spherisorb ODS2 column (25.0 cm×0.46 cm, 5 μm particle size; Waters, Milford, MA, USA) maintained at 26 °C. The mobile phase solvents consisted of 1% (v/v) formic acid in water (eluent A) and methanol (eluent B) and a gradient program was used as follows: from 10 to 15% B (5 min), from 15 to 40% B (20 min), from 40 to 55% B (25 min), from 55 to 100% B (10 min), 100% B isocratic (20 min) run at a flow rate of 0.4 mL/min. Spectral data from all peaks (200-600 nm) and chromatograms (280, 320, 330 nm) were collected using an Agilent 1100 series diode array detector. Retention times and UV-vis spectra of authentic standards analysed in the same conditions were used for the identification of phenolic compounds. For quantification purposes, the extract was injected in triplicate. The amount of phenolic compounds present in the extract

was achieved from the calibration curves of the respective authentic standard analysed under the same conditions (Table 1).

The linearity range of the method was assessed by building calibration curves using, at least, six different concentration levels of the analytes, according to the range of concentrations found in the sample. The limit of detection (LOD) and limit of quantification (LOQ) were determined from calibration curve data, following the formula:

$LOD = (3 \times SD)/b$

$LOQ = (10 \times SD)/b$,

where *SD* is the residual standard deviation and *b* is the slope. The repeatability and reproducibility of the method were calculated in terms of intra-day and inter-day precision, respectively. Repeatability was performed by injecting the *J. glutinosa* extract, by the same analyst, 5 times in the same day, whereas, reproducibility was performed by injecting that same sample in triplicate during 5 consecutive days. Final results were expressed as coefficient of variation (CV, %) (Table 1).

2.3.1.2. Pigments (carotenoids and chlorophylls) determination

20 µL of re-dissolved and filtered ethanolic extract was analysed using a Gilson HPLC unit with a C30 YMC carotenoid column 5 µm, 250 × 4.6 mm (YMC, Japan) maintained at 26 °C, following the procedure described by Amaro *et al*., (Amaro et al. 2015) with some modifications. The mobile phase was composed by two solvents, methanol (A) and tert-butyl methyl ether (B); elution started with 95% A and used a gradient to obtain 70% at 30 min, 50% at 50 min, 0% at 60 min, 0% isocratic at 65 min and 95% at 70 min. In this case, spectral data from all peaks (200-700 nm) and chromatograms at 450 nm were collected using an Agilent 1100 series diode array detector. The data were processed with Clarity Software, version 5.04.158 (DataApex Ltd, Prague, Czech Republic). Peak purity was checked by the software contrast facilities. The extract was injected in triplicate. The amount of pigments was calculated using external calibration curves. Only the compounds, for which we had reference standards, were quantified. The linearity range, the LOD and LOQ, the repeatability and reproducibility of the method (Table 1) were determined according to the one described above.

2.4. Cell-free assays

2.4.1. Antioxidant activity

2.4.1.1. Non-enzymatic assay

According to a procedure previously documented, the NADH/PMS system was used for the generation of superoxide radicals (Valentão et al. 2001). Due to the low solubility of the extract in the phosphate buffer (19 mM, pH 7.4), we used a non-interfering DMSO percentage (1% *v/v*, phosphate buffer). The remaining components were dissolved in phosphate buffer. Antiradical activity was determined spectrophotometrically at 562 nm, in a 96-well Multiskan Ascent plate reader (Thermo; electron corporation), working in kinetic mode, by monitoring the effect of extract on reduction of NBT induced by superoxide radical. Four independent assays were performed in triplicate and quercetin as positive control.

2.4.1.2. Enzymatic assay

The xanthine/xanthine oxidase (X/XO) system was used for the enzymatic generation of superoxide radicals (Rodríguez-Chávez et al. 2015). The assay was performed in 96-well microplates, and the mixture contained 90 µM xanthine, 16 mM Na_2CO_3 and 22.8 µM NBT dissolved in phosphate buffer (pH 7). Then,

30 µL of sample at different concentrations (0.0125, 0.0039, 0.0020, 0.0010, 0.0005, 0.0002, 0.0001, 0.00006, 0.00003 mg/mL) and 30 µL of 168 U/L xanthine oxidase were added to start the reaction. Incubation time: 2 min at 37 °C. Absorbance was measured at 560 nm. Gallic acid was used as reference.

The effect on xanthine oxidase was also evaluated by measuring the formation of uric acid from xanthine at 295 nm after 2 min. The reaction mixture contained the same components as described above in the xanthine/xanthine oxidase system but the reaction mixture did not contain 22.8 µM NBT.

2.4.2. Anti-inflammatory activity

2.4.2.1. 5-Lipoxygenase inhibition

The inhibitory effect on 5-LOX was assessed in 96-well plates, using a procedure previously described by Barbosa *et al*., (Barbosa et al. 2017). A Synergy™ HT microplate reader (Biotek Instruments; Winooski, USA) was used to detect 5-LOX inhibitors at 234 nm for 3 min. 5-LOX inhibition was calculated as follows: LOX inhibitory activity (%) = 100 × [1− (mean V of sample/mean V of control)], where mean V corresponds to the mean velocity of kinetic well analysis. Six independent assays were performed in triplicate. Quercetin was used as positive control.

2.5. Cell-based assays

2.5.1. Cell culture conditions

Murine macrophages (J774.2; The Agrifood Research and Technology Centre of Aragon; Zaragoza, Spain) were cultured in 96-well plates with Roswell Park Memorial Institute 1640 Medium (RMPI 1640 medium, GlutaMAXTM Supplement) containing 10% Fetal Bovine Serum, 100 U/mL penicillin G and 100 µg/mL streptomycin. Cells were seeded in 96 well microplates at a density of 10^3 cell/well and incubated at 37 °C in a humidified incubator.

2.5.2. NO levels

Nitrite content in culture medium was measured as an indicator of nitric oxide (NO) production. Nitrites were measured at 24 h after the addition of different concentration of RT extract (23, 47 or 93 µg/mL) and LPS (1 µg/ml) for 30 minutes. The same volume of supernatant and the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed during 10 minutes at room temperature and the absorbance at 570 nm was determined with a plate reader.

2.5.3. TNF-α levels

For determination of tumour necrosis factor-alpha (TNF-α) levels, cells were incubated with various concentrations of RT extract (23, 93 µg/mL) and LPS (1 µg/ml)-stimulated for 30 minutes. After centrifugation, the obtained supernatant was used to calculate the amount of TNF-α by a standard sandwich ELISA kit specific for murine cytokines (Cayman chemical®).

2.6. *In vivo* assays

2.6.1. Animals

Male C57BL/6 mice (8-12 weeks) were purchased from Janvier (LeGenest St. Isle, France) and were allowed to acclimate for 1 week prior to the experiments. The animals were housed in plastic cages, 4-5 mice/cage, under normal laboratory conditions (20 ± 2 °C, 40-60% relative humidity and normal light/dark-cycle) with free access to standard chow and water.

All the procedures of this study were performed in accordance with the ARRIVE guidelines and the Ethics Advisory Commission for Animal Experimentation of Universidad de Zaragoza (PI66/14, Spain).

2.6.2. Treatment, experimental colitis induction and tissue collection

Animals were randomly divided into seven groups. i) control group (n=15); ii) RT_{50} group (n=8); iii) water+DSS group (n=15); iv) RT_5+DSS group (n=4); v) RT_{25}+DSS group (n=4); vi) RT_{50}+DSS group (n=15) and vii) sulfasalazine (SSZ) + DSS group (n=12). All groups daily received 200 µL of water (control or water+DSS groups), RT extract (5, 25 or 50 mg/kg bw: RT_5+DSS groups, RT_{25}+DSS groups, RT_{50} or RT_{50}+DSS groups) or SSZ (100 mg/kg bw) by oral gavage over a 20-day period according to Castro et al., 2016 (Castro et al. 2016). The dose of SSZ has been chosen according to previous studies (Kim et al. 2014; Singh et al. 2009). RT and SSZ solutions were prepared daily. In order to avoid the undesirable effects of coprophagy, all the animals housed in each cage received the same treatment.

An injury-repair colitis model was used (Whittem et al. 2010). At day 11, colitis was induced by switching to a 2.5% (w/v) solution of DSS (MW: 40,000 Da, Panreac, Lörrach, Germany) as drinking solution for 7 days, followed by 3 days of autoclaved tap water for the recovery (Low et al. 2013). At day 20, all animals were sacrificed via cervical dislocation and the colon was removed for macroscopic study (Figure 3a). Terminal ileum was extracted and kept in cold Ringer Krebs (in mM: NaCl 120, KCl 4.70, $CaCl_2$ 2.40, $MgSO_4$ 1.20, $NaHCO_3$ 24.50, KH_2PO_4 1.00 and glucose 5.60; pH 7.4) for organ bath studies. Finally, samples of colon of control, RT_{50} group, water+DSS and RT_{50}+DSS animals were kept for histopathological, immunofluorescence, myeloperoxidase (MPO), interleukins 6 and 10 (IL-6, IL-10), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) studies.

2.6.3. Colitis assessment

A disease activity index (DAI) was used to assess the DSS-induced colitis symptomatology. DAI was calculated as the sum of the body weight score, which was the percentage of weight loss from the initial body weight on every day (0: < 1%, 1: 1-5%, 2: 5-10%. 3: 10–15%, 4: ≥15%), changes in stool consistency (0: normal, 1: loose stool, 4: water diarrhoea) and presence of faecal blood (0: normal, 2: occult bleeding, 4: visible bleeding). The occult blood was measured with a stool blood test (Tri Slider FOB Guayaco, Sanilabo, Spain).

2.6.4. Macroscopic study

Colon length and thickness of the proximal and distal colonic wall were determined using a digital Vernier calliper (0.01 mm of resolution). Thereafter, the colon was cut-open longitudinally and washed with 0.9% saline solution. Colitis signs was graded according to a standard scoring system: Number of adhesions (0: 0, 1: 1, 2: 2, 3: >2), diarrhoea (0: normal, 1: loose stool, 4: water diarrhoea), tumefaction (0: absence, 1: moderate, 2: severe), stenosis (0: 0, 1: 1, 2: 2, 3: >2), mucus (0: absence, 1: presence), haemorrhage (0: absence, 1: manifest bleeding), erythema (0: no macroscopic changes, 1: <1 cm, 2: ≥1 cm), ulcers or erosions (0: 0, 1: <1 cm, 2: ≥1 cm) and occult blood (0: no, 1: yes). A total macroscopic damage score was calculated for each animal.

2.6.5. Recording of intestinal contractility

Ileum was washed with Ringer Krebs, freed from adipose and connective tissues and cut in segments (10 mm). The whole segments were suspended in the direction of longitudinal smooth muscle fibres in an organ bath in 5 mL of Ringer Krebs maintained at 37 °C and gassed with 95% O_2-5% CO_2. Intestinal contractility was recorded isotonically as in previous works of our laboratory (Valero et al. 2011).

After an adaptation period, spontaneous contractions of each group were registered and the effect on contractility of acetylcholine (ACh, 100 µM), a cholinergic agonist, was tested. To measure the irregularity of contractions the mean CV of each longitudinal contraction was estimated. During a period of 5 minutes, the mean of amplitude and frequency of spontaneous contractions were analysed. ACh-induced motor responses were measured as the area under the curve (AUC) during the first 3 min of ACh response and expressed as a percentage of control (3 min of the AUC area before addition of ACh). The AUC was normalized by the wet weight of each preparation. Results were expressed as a percentage of control group values (100%).

Each experimental protocol was systematically made on one segment of ileum per mouse and was repeated on 4-10 animals.

2.6.6. Histopathological study

Pieces of proximal and distal colon were fixed in 10% buffered formalin (Panreac, Barcelona, Spain), then dehydrated, embedded in paraffin blocks, and cut into 4 µm-thick sections. They were stained with haematoxylin-eosin (HE). The analysis of these samples was performed by a pathologist by measuring the thickness of the intestinal mucosa in 5 different areas using a Nikon Eclipse Ci microscope, a Nikon DS-Ri 1 digital still camera, and a Nikon NIS calibrated digital image analysis system. Histopathological analysis focused on normality or alteration of architecture and relative proportions of the structures, presence of abnormal vascularization, assessment of degenerative or necrotic features, degree of inflammation (cell infiltrates), and abnormal growth (atrophy, hyperplasia, hypertrophy and neoplasia).

2.6.7. Determination of MPO activity

Samples of distal colon stored at -80 °C were homogenized in 0.1 M phosphate buffer (pH 7.4) and then centrifuged at 15.000 rpm at 4 °C for 10 min, the supernatant fraction was used for the measurements of MPO content by the MPO activity Assay Kit (Sigma Aldrich). MPO activity was expressed as U/mg protein.

2.6.8. Determination of IL-6 and IL-10

Levels of IL-6 and IL-10, pro- and anti-inflammatory interleukins, respectively, were measured in colon homogenates (1:5 dilution) and samples of cells (1:2 dilution) using ELISA kits (KIT Ready-Set-Go!, eBioscience) as per the manufacturer's instructions.

2.6.9. Protein extraction and Western blot analysis for determination of iNOS and COX-2

Samples of distal colon were homogenized in cold lysis buffer containing 150 mM NaCl, 10 mM Tris pH 7.5 and 1% Triton X-100 supplemented with a complete EDTA-free protease inhibitor cocktail (Roche). The homogenates were centrifuged at 15.000 rpm at 4 °C for 20 min, and the protein concentration of the supernatants was assessed using a Pierce BCA Protein Assay Kit (Thermo-Fisher).

Proteins were separated on 7.5% SDS–PAGE gels (Bio-Rad) and transferred onto PVDF membranes (Immobilon-P; Millipore). The membranes were blocked with 5% non-fat milk in Tris buffered saline supplemented with 0.1% Tween-20 (TBST) at room temperature during 2h. After that, membranes were incubated with primary antibodies for iNOS (1:200, BD Biosciences), COX-2 (1:1000, BD Biosciences) and β-actin (1:5000, Santa Cruz Biotechnology, as loading control) overnight in TBST with 1% non-fat milk at 4 °C. Membranes were then washed and incubated with peroxidase-conjugated secondary antibodies in TBST with 1% non-fat milk for 2h at room temperature. The immunocomplexes were detected by a

chemiluminescent reaction using a Western Bright Sirius (Advansta) kit and the signal was visualized in VersaDoc (Bio-Rad). Results were expressed as iNOS/actin or COX-2/actin ratios in arbitrary densitometric units.

2.6.10. Immunofluorescence and confocal microscopy

Proximal colon sections were deparaffinised through Histo-Clear (National Diagnostics), and rehydrated in decreasing concentrations of ethanol and rinsed with distilled water and Tris buffered saline (TBS; 65 mM Tris-Cl, pH 7.5; 150 mM NaCl). Tissues were treated for 30 minutes with sodium borohydride ($NaBH_4$; 1% w/v) in ice-cold TBS to reduce auto-fluorescence. Non-specific binding was blocked with 1% BSA and 0.5% Triton X100 in TBS (blocking buffer) for 3x10 minutes. Then, slices were incubated at 4 °C overnight with a goat anti-ZO-1 antibody (1:50, Santa Cruz Biotechnology). The next day, sections were washed with blocking buffer (3x10 minutes) and incubated with a donkey anti-goat IgG-AlexaFluor 488 secondary antibody (1:2000, Molecular Probes) in darkness for 2 h at room temperature. After 3 washes for 15 minutes each in TBS, preparations were mounted with Fluoromount-G (SouthernBiotech) containing 0.1% DAPI (Life Biotechnologies) and 0.4% DraQ5 (BioStatus) for DNA staining, and visualized under a confocal microscope (Olympus FV10). The images were acquired using the Olympus FV10-ASW 3.0 viewer software and further processed with ImageJ.

2.7. Statistical analysis

Data are expressed as mean ± SEM, and differences between groups were statistically analysed using the GraphPad Prism Software v6. One-way analysis of variance (ANOVA) followed by Tukey's (when parametric distribution is observed), Kruskal Wallis tests (non parametric) or unpaired t-tests were used to detect differences. Parameters of spontaneous contractions (amplitude and frequency) were analysed as previously described on duodenum segments (Fagundes et al. 2007). Significance level was set to $p < 0.05$.

3. Results

3.1. Phytochemical analysis

3.1.1. Phenolic compounds

The HPLC-DAD analysis of the extract revealed the presence of fifteen phenolic compounds, including ten phenolic acids (**1**-**9**, **12**) and five flavonols (**10**, **11**, **13**, **14** and **15**) (Figure 1a). The ethanolic extract herein studied showed a high content of phenolic compounds (134.4 mg/g of dry extract) (Table 1). Representing more than 70% of all phenolics quantified, the hydroxycinnamic acids derived from caffeoylquinic acid were the dominant compounds in the extract, the pair 3,4-di-*O*-caffeoylquinic and 3,5-di-*O*-caffeoylquinic acids (**7**+**8**) being dominant (40.95 mg/g of dry extract), followed by 1,5-di-*O*-caffeoylquinic (**9**) and 4,5-di-*O*-caffeoylquinic (**12**) acids (24.73 and 23.14 mg/g of dry extract, respectively) (Table 1). The flavonol quercetin-3-*O*-galactoside (**10**) was the fourth most representative compound in the extract (15.16 mg/g of dry extract) and the most abundant flavonoid found.

3.1.2. Pigments

The analytical methodology employed allowed the determination, for the first time, of two carotenoids, comprising one xanthophyll, lutein (**17**), and β-carotene (**23**), as well as of chlorophyll *b* (**6**), the chlorophyll *a* demetalation derivative pheophytin *a* (**22**), and compounds with chlorophyll *a*-like UV-vis spectrum, labelled as chlorophyll *a* derivatives (**20** and **21**). Two other compounds (compounds **18** and **19**) were also

found; although their identity is not known, their chromatographic behaviour and UV-vis spectra allow classifying them as xanthophylls (Figure 1b). Among the carotenoids and chlorophylls quantified in our study, lutein (**17**) was the most representative, being its content higher than 55% of total pigments (Table 1).

3.2. Antioxidant potential in cell-free assays

RT extract showed an effective and dose-dependent antioxidant effect against $O_2^{\bullet-}$ radicals generated by X/XO system, presenting an IC_{50} of 36.7 μg/mL. An IC_{50} value of 0.46 μg/mL was obtained for gallic acid, the positive control tested under the same conditions (Figure 2a).

Moreover, RT extract, at higher doses, was also able to inhibit the xanthine oxidase enzyme responsible for this reaction, with an IC_{50} value of 929.86 μg/mL (Figure 2b).

The capacity of the RT extract to scavenge superoxide radical generated by a non-enzymatic system was confirmed when this radical was generated by a chemical system, which indicated an IC_{50} of 47.0 μg/mL (Figure 2c). An IC_{50} value of 10.97 ± 0.68 μg/mL was obtained with quercetin, the positive control tested under the same conditions. This fact allows to conclude that the superoxide scavenging properties are independent to xanthine-oxidase inhibition.

3.3. Anti-inflammatory activity

RT extract also inhibited 5-LOX, showing an IC_{50} of 74.0 μg/mL (Figure 2d). An IC_{50} value of 3.29 ± 0.21 μg/mL was obtained for quercetin, the positive control tested under the same conditions.

LPS (1 μg/mL) increased the nitrite production and TNF-α expression in murine macrophages compared with control. RT extract presented a significant capacity to reduce to basal levels both NO production as well as the expression of the pro-inflammatory cytokine TNF-α in LPS-stimulated J774.2 macrophages (Figure 2e and 2f). These results confirmed the anti-inflammatory potential of RT extract.

3.4. Protective effects of the RT extract on the development and recovery of DSS-induced colitis.

RT *per se* did not affect clinical state of animals, as shown in Figure 3b. Colitis was manifested by weight loss, presence of blood in faeces, and changes in stool consistency. The DAI scores in Figure 3b showed that the RT extract, to different doses, delayed the appearance of colitis symptoms. However, at longer DSS-exposure (≥4 days), this protective effect vanished. Yet, the RT extract improved symptoms during the recovery phase of the animals. Thus, total disease severity was found to be lower in mice treated with RT extract vs. untreated colitic animals. SSZ, used as standard treatment of UC, was also effective in the initial phase, although less than RT (Figure 3b and 3c). In contrast to RT, SSZ did not improve disease severity in the recovery period (day 20) (Figure 3c).

3.5. RT extract attenuates macroscopic colon damage in DSS-induced acute colitis.

DSS administration reduced colonic length in all groups (Figure 4a). Administration of RT_{50} extract but not the reference drug SSZ significantly attenuated this effect. Also, wall thickness was increased in both proximal and distal colon after DSS. RT, dose-dependently, and SSZ mitigated this effect (Figure 4c and 4d), although only reached statistical significance the effect of SSZ in proximal colon. Finally, DSS-induced macroscopic damage, as seen in Figure 4b, was partially reversed by the RT extracts and SSZ. The administration of RT extract in animals that were not challenged with DSS did not produce any pathological alterations suggesting that the RT is not toxic at the dosage used here (Figure 4a-d).

3.6. Functional studies of ileal contractility

Figure 5a shows the typical spontaneous contractile activity of longitudinal muscle segments of ileum isolated from different groups (control, RT_{50}, water+DSS, RT_5+DSS, RT_{25}+DSS, and RT_{50}+DSS group). RT_{50} extract *per se* did not change motility pattern. As expected, DSS severely impaired spontaneous contractions. Thus, as shown in Figure 5b, the longitudinal smooth muscle contractions were highly irregular and intermittent over time. In contrast, spontaneous contractions of the ileum segments from the RT-treated animals showed dose-dependent improvement of rhythm, being very similar to control with the higher doses of the plant ($p=0.0095$ water+DSS vs. RT_{25}+DSS and $p=0.0061$ water+DSS vs. RT_{50}+, in a Mann-Whitney test). Figure 5c shows that the DSS treatment also significantly decreased the amplitude of spontaneous contractions if compared to control, except for RT_{50}+DSS-treated animals ($p=0.0016$ water+DSS vs. RT_{50}+DSS, Mann-Whitney test). The DSS treatment mildly reduced the frequency of ileal spontaneous contractions but the difference did not reach statistical significance (Figure 5d).

The DSS treatment significantly decreased the contractions to ACh. Again, RT extract at higher but not lower doses prevented this effect (Figure 5e). Taken together, these results show that the RT treatment, in a dose dependent way, was able to ameliorate DSS effects on ileal motility and contractile capacity.

The highest dose of RT was chosen for the rest of experiments due to its better effects on the clinical symptomatology, tissue damage, and functional studies.

3.7. RT extract ameliorates histopathological alterations in DSS-induced colitis.

Colon sections of DSS-treated animals showed severe colitis with massive destruction of the intestinal mucosa and severe infiltration of inflammatory cells into the lamina propria, especially in the distal colon (Figure 4f). In contrast, DSS-induced damage and inflammation was significantly lower in the distal colon in RT_{50}-treated animals (Figure 4g), while the damage in the proximal colon was similar. The controls and the RT_{50}-treated animals showed a typical colon structure, with normal sized intestinal villi, a normal lamina propria and submucosa with mild cell infiltrates (Figure 4e and 4h).

3.8. Biochemical markers of inflammation (MPO, IL-6, IL-10, iNOS, COX-2) in colonic tissue

MPO activity and IL-6 levels were significantly higher in colonic tissues of the DSS group than in controls. RT_{50} extract significantly reduced the DSS effect (Figure 6a and 6b). Colon of animals treated with DSS showed a decrease in the levels of IL-10 respect to controls ($p = 0.057$), an effect that was not prevented by the treatment with RT_{50} extract (Figure 6c).

The effect of RT on inflammation was also studied by assessing the protein expression of iNOS and COX-2. As shown in Figure 6d and 6e, as expected, the expression levels of iNOS and COX-2 were significantly increased in colonic tissues of DSS group respect to control. RT_{50} extract did not induce *per se* expression of COX-2 or iNOS but it significantly prevented the induction of these enzymes by DSS.

3.9. RT extract prevents DSS-induced loss of ZO-1.

ZO-1, a tight junction protein of the intestinal epithelial barrier, was visualized by immunofluorescence / confocal microscopy in colon samples of control, water+DSS and RT_{50}+DSS groups.

As shown in Figure 6a, ZO-1 was mainly expressed at the apical border of the lining epithelium in control animals. Furthermore, a dotted-spherical pattern of expression, likely cytoplasmic, was also observed deeper in the tissue (Figure 7a). In contrast, in 3 out of 3 DSS mice apical ZO-1 expression was lost, while the dotted-spherical expression was preserved (Figure 7b). The treatment with RT_{50} extract prevented the loss of apical ZO-1 in the lining epithelium of DSS-challenged animals in 3 out of 3 animals (Figure 7c).

4. Discussion

This study characterized phytochemically an ethanolic extract of RT and investigated its ability to improve experimental DSS-induced colitis in mice by using a wide range of cell-free, cell-based, *in-vivo* and *ex-vivo* techniques. Data demonstrate that RT was capable of ameliorating clinical symptoms of colitis in this model, i.e., weight loss, loose and bloody stool, inflammation, damage of the colon mucosa, including the preservation of the intestinal barrier. Moreover, the extract has been shown to normalize the severely impaired ileal contractility observed in this model. Anti-inflammatory and antioxidant effects have also been detected, which could explain the observed *in-vivo* benefits. It is the first time that a study provides experimental proof for the utility of *Jasonia glutinosa* as herbal medicine or herbal supplement in UC.

RT extract is rich in polyphenols that are well-known antioxidant agents. Some of them have been described to possess *in-vitro* anti-inflammatory activities (Hussain et al. 2016). A recent study simulating an *in vitro* digestion showed that digestive processes may alter the composition of *Jasonia glutinosa* extracts (Ortega-Vidal et al. 2019); however, our extract preserved its anti-inflammatory properties both in *in vitro* and *in vivo* assays.

RT extract showed a rich and varied phenolic profile, composed of flavonols and hydroxycinnamic acids, predominantly caffeoylquinic acids (Figure 1a, Table 1). Previous phytochemical studies have shown the presence of flavonoids, like quercetin, kaempferol derivatives and phenolic acids in methanolic and aqueous extract of RT (Ortega-Vidal et al. 2019; Rubio et al. 1995; Valero et al. 2013). We here show that ethanolic extract is also rich in phenolic acids, in particular caffeoylquinic acids, and pigments, which could be of interests for industrial purposes as ethanol (instead of methanol as solvent) is permitted and used in pharmaceutical products. Other studies have found kaempferol in RT extracts, as well as some glycosides of quercetin, such as quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside. Oral administration of high amounts of isolated flavonoids also showed beneficial effects on DSS-induced colitis in mice as they prevented weight loss, histological alterations and inflammation caused by colitis (Brückner et al. 2012; Camuesco et al. 2004; Comalada et al. 2005; Park et al. 2012). Probably, these actions could be mediated through down-regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), that results in a reduction of pro-inflammatory cytokines as IL-6, IL-1β and TNF-α, iNOS expression and NO levels, LOX and COX-2 expression, prostaglandin E_2 levels and MPO activity (Brückner et al. 2012; Camuesco et al. 2004; Comalada et al. 2005; Liu et al. 2014; Park et al. 2012).

Concerning caffeoylquinic acids found in our RT extract, it is worth mentioning that esters formed by caffeic and quinic acids (caffeoylquinic acids), represent an abundant group of plant polyphenols present in human diet and their role in controlling oxidative and inflammatory stress conditions is reviewed in previous works (Liang and Kitts 2015). The anti-ulcerogenic properties of some caffeoylquinic acids, such as 5-*O*-caffeoylquinic, 3,4-di-*O*-caffeoylquinic and 3,5-di-*O*-caffeoylquinic acids has already been documented (Lee et al. 2010). In several animal studies, the oral administration of 5-*O*-caffeoylquinic acid showed protective effects in different induced-colitis models, by reducing neutrophil infiltration and inhibition of the NF-κB pathway (Liang and Kitts 2015) and by promoting growth of *Akkermansia* in mice (Zhang et al. 2017).

Carotenoids represent the most abundant lipid-soluble phytochemicals, and *in-vitro* and *in-vivo* studies have suggested their antioxidant and anti-inflammatory properties (Kaulmann and Bohn 2014). The problems observed in the digestion and absorption of fat-soluble compounds in patients with IBD could delay their protective effects, as they are described against colon related diseases, such as cancer, Crohn disease and UC (Slattery et al. 2000).

Two biologically important carotenoids were found in our RT extract, lutein (**17**) and β-carotene (**23**). Glabska and colleagues reported that a higher intake of a mixture of lutein and zeaxanthin (ca. 1800 µg) was associated with the reduction of faecal blood, mucus and pus in individuals with UC (Głąbska et al. 2016), and with lower incidence of constipation in individuals with UC in remission phase (Głąbska et al. 2019). Likewise, β-carotene (at doses of 5-50 µM) was described to ameliorate UC-associated local and systemic damage in mice, by acting on multiple targets (Kaulmann and Bohn 2014; Trivedi and Jena 2015). RT extract presented a lutein content (ca. 148 µg/g) almost eight times higher than that of β-carotene (ca. 20 µg/g) (Table 1). Although in the work herein a daily content of 50 mg RT of extract/kg bw was administrated to the animals and, at this doses, the concentration of lutein and β-carotene (ca. 7.4 µg and 1 ng, respectively) were much lower than those showing therapeutic efficacy (Głąbska et al. 2019; Głąbska et al. 2016; Kaulmann and Bohn 2014; Trivedi and Jena 2015), the possibility of contribution of these compounds as themselves, as well as of the synergic effects between them, cannot be ignored.

Concerning the underlying mechanisms by which RT shows therapeutic efficacy in DSS-induced colitis, our *in-vitro* studies revealed that RT extract strongly scavenged superoxide radicals using the X/XO system. However, when using this assay, we cannot fully exclude a direct inhibitory effect on the enzyme itself. Indeed, we found that the extract presented a low XO inhibitory activity (IC_{50} = 929.86 µg/mL). Still, when the extract was tested in the NADH/PMS system, we found a clear-cut $O_2^{\bullet-}$ scavenging effect.
Respecting the anti-inflammatory properties of RT in the DSS model, we found that RT inhibited 5-LOX-activity, which is known to be required for the synthesis of pro-inflammatory molecules, i.e. leukotrienes. Among several isoforms, 5-LOX is one of the most important in physiological and pathological processes (Rådmark et al. 2015) and LOX-inhibitors have been found in plant extracts (Schneider and Bucar 2005). Therefore, the lower degree of inflammation in RT-treated mice can be explained through 5-LOX inhibiting properties. In addition, RT extract reduced the production of NO and TNF-α induced by LPS in J774.2 macrophages. In colonic tissue, RT extract decreased MPO activity, the rise in IL-6 levels as well as in COX-2 and iNOS expression. On the other hand, RT extract had no effect on IL-10 levels. Together, these findings suggest that RT has potent and multifaceted anti-inflammatory properties involving several signal pathways and cell systems. Interestingly, this anti-inflammatory profile shares similarities with anti-inflammatory properties of other medicinal plant compounds, such as corilagin, a TGFβ1-inhibiting molecule (Xiao et al. 2013).

Our immune histological studies suggest that the efficacy of this extract also relied on maintaining the integrity of intestinal barrier intact, as concluded from the conserved ZO-1 expression at enterocytes tight

junctions. Intact functioning of tight junctions is essential for building the intestinal barrier (Mankertz and Schulzke 2007; Poritz et al. 2007). In addition, it is well known that cytokines regulate tight junctions (Capaldo and Nusrat 2009) and, particularly, TNFα and IL-6 have been reported to impair intestinal barrier by interfering with ZO-1 protein expression and localization (Desai et al. 2002; Ma et al. 2004). The protective effect of RT on the intestinal barrier may therefore be explained by RT's ability to prevent TNFα and IL-6 induction.

Regarding appearance and progressive aggravation of clinical symptoms, RT was also effective. However, this therapeutic effect was seen during the first three days of the DSS treatment but not during the next four days. Additionally, RT significantly promoted recovery after cessation of the inflammatory stimulus. If compared this efficacy with that of a standard anti-inflammatory treatment for UC, i.e. SSZ, RT showed similar or even better efficacy considering that RT but not SSZ delayed the development of severe symptomatology and promoted recovery. At the anatomical/histological level, RT extract prevented to a considerable extent the reduction of colonic length and the increase in the wall thickness that are typical features of this DSS model. It is worth mentioning that studies testing green tea polyphenols (Oz et al. 2013) or foods rich in polyphenols (Kang et al. 2017; Kim et al. 2014; Singh et al. 2009) and also using SSZ as standard treatment in this colitis model, showed comparable therapeutic efficacy.

Besides morphological and histological changes, it is known that DSS impairs motility in the colon and, more recently, also in the small intestine (Lykov et al. 2018; Yazbeck et al. 2011). Here, we provide additional evidence for this harmful impact of DSS on ileal contractility by showing un-coordinated and weaker spontaneous contractions and weaker ACh-induced responses. Importantly, we show that RT, in a dose dependent manner, was also capable of preventing this alteration. Thus, RT_{50}-treated mice exhibited coordinated rhythmic spontaneous motility and conserved amplitude of contractions. This findings are also in line with our previous work showing that RT at 50 mg/kg normalized whole gastrointestinal transit in this model of colitis (Castro et al. 2016). This protective effect of RT on ileal motor function may be due to a decrease on local inflammatory mediators as shown with other vegetal extracts (Chaudhuri et al. 2000). This beneficial effect on intestinal motility is likely the functional outcome of RT´s complex anti-inflammatory, antioxidant and tissue-protective effects.

5. Conclusions

In summary, this work provides evidence that RT extract has beneficial effects in a DSS-induced colitis model, improving clinical symptoms, attenuating tissue damage, preserving the intestinal barrier and normalizing the intestinal contractility. The benefits of RT extract could be related to its biologically-relevant chemical composition. We suggest that RT may be of potential therapeutic utility in UC and other inflammatory bowel diseases.

Declarations

Ethics approval and consent to participate: This study was performed in accordance with the European Union Directive 2010/63 EU concerning experimental animal protection. Experimental protocols were approved by the Ethics Committee of Universidad de Zaragoza.

Consent for publication: All authors gave their informed consent prior to their inclusion in the study.

Conflict of interest: The authors declare that they have no conflict of interest.

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Figure captions

Figure 1. Representative HPLC-DAD chromatogram of the (a) phenolic compounds and (b) pigment profiles of *J. glutinosa* extract. Detection at 320 and 450 nm, respectively. (**1**) 3-*O*-caffeoylquinic acid; (**2**) 5-*O*-caffeoylquinic acid; (**3**) 4-*O*-caffeoylquinic acid; (**4**) caffeic acid; (**5**) 1,3-di-*O*-caffeoylquinic acid; (**6**) Isoferulic acid; (**7**) 3,4-di-*O*-caffeoylquinic acid; (**8**) 3,5-di-*O*-caffeoylquinic acid; (**9**) 1,5-di-*O*-caffeoylquinic acid; (**10**) quercetin-3-*O*-galactoside; (**11**) quercetin-3-*O*-glucoside; (**12**) 4,5-di-*O*-caffeoylquinic acid; (**13**) quercetin; (**14**) kaempferol; (**15**) isorhamnetin; (**16**) chlorophyll *b*; (**17**) lutein; (**18** and **19**) unidentified xanthophylls; (**20** and **21**) chlorophyll *a* derivatives; (**22**) pheophytin *a*; (**23**) β-carotene.

Figure 2. Antioxidant activities and anti-inflammatory effect in cell-free and cell-based assays. a) Antioxidant activity of RT extract and gallic acid against superoxide radicals generated by the xanthine/xanthine oxidase method. b) Xanthine oxidase inhibition by RT extract. c) Effect of *J. glutinosa* extract against $O_2^{\bullet-}$ generated by the NADH/PMS system. d) Enzymatic inhibition of *J. glutinosa* extract towards 5-LOX. e) Effect of RT extract on production of nitrate concentration in LPS-stimulated cells (1 µg/ml). f) Inhibitory effect of RT in TNF-α expression in LPS-stimulated cells (1 µg/ml). Results show mean ± SEM of four or six experiments, each performed in triplicate. $^{**}P<0.001$ vs. control group, $^{\#}P<0.05$ vs. LPS in absence of RT.

Figure 3. Induction and symptomatology of colitis. a) Experimental design of the induction of colitis by DSS administration for 7 days. Mice were daily treated with either RT extract (5, 25 or 50 mg/kg bw, p.o.), sulfasalazine (SSZ, 100 mg/kg bw), or vehicle over 20 days. From day 11 to day 17, mice received 2.5% dextran-sodium sulphate (DSS). At day 18, the drinking water with DSS was removed and three days of recovery were evaluated. b) The DAI score was assessed daily in all groups. Data are presented as mean ± SEM (n=4-15). $^{*}P<0.05$, $^{***}P<0.001$ water+DSS group vs. control and RT_{50} groups; $^{\&}P<0.05$ RT_5+DSS group vs control and RT_{50} groups; $^{\$}P<0.05$ RT_{25}+DSS group vs control and RT_{50} groups; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ RT_{50}+DSS group vs control and RT_{50} groups; $^{\pi}P<0.05$, $^{\pi\pi\pi}P<0.001$ SSZ+DSS group vs. control and RT_{50} groups. c) Comparison of the protective effect against colitis between RT_{50} and SSZ. Data are presented as mean ± SEM (n=8-15). $^{*}P<0.05$, $^{**}P<0.01$ RT_{50}+DSS group vs. water+DSS group; $^{\varepsilon\varepsilon}P<0.01$ RT_{50}+DSS vs. SSZ+DSS group.

Figure 4. Beneficial effects of RT extract on macroscopic and histopathological changes in the colon. a) Colon length. b) Macroscopic damage score. c) Proximal colon thickness. d) Distal colon thickness. Data are presented as mean ± SEM (n=4-15). $^{*}P<0.05$, $^{***}P<0.001$ vs. control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. RT_{50} group; $^{\&}P<0.05$, $^{\&\&}P<0.01$ vs. water+DSS group. e-h) Images of HE staining of distal colon on different groups: e) control group, f) water+DSS group, g) RT_{50}+DSS group, h) RT_{50} group (n=4). Scale bars represent 50 µm (e, h) or 100 µm (f, g).

Figure 5. Effects of RT extracts on alterations in intestinal contractility observed in colitis. a) Recordings of spontaneous contractions from longitudinal smooth muscle of mice ileum in different groups. b) Mean coefficient of variation of each longitudinal contraction in the ileum. c) Percentage of amplitude and d) frequency on spontaneous contractions. e) Effect of ACh (100 µM) on spontaneous contractions. Data are expressed as mean ± S.E.M. Segments obtained from 4-10 mice. $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. RT_{50} group

Figure 6. RT extract attenuates the inflammation and oxidant effect of DSS in colon from colitic mice. a) Colonic MPO activity, b) IL-6 levels and c) IL-10 levels in DSS-induced colitis. Representative western blots and quantitative analysis of d) iNOS protein and e) COX-2 protein. Data are presented as mean ± SEM (n=6-8). $^{*}P<0.05$, $^{**}P<0.01$ vs. control group; $^{\&}P<0.05$, $^{\&\&}P<0.01$ vs. water+DSS group.

Figure 7. Effect of RT extract on the expression of the tight junction protein ZO-1. Representative confocal images (n=3) on a) control group, b) water+DSS group and c) RT_{50}+DSS group. In control mice (a), ZO-1 was expressed at the luminal part of the epithelium (arrows) but an inner spherical staining was also observed (arrowheads). DSS decreased apical expression, effect reverted by RT_{50} extract administration. M: mucosa. L: lumen.

788 **Table 1.** Phenolics and pigments content of *J. glutinosa* extract (mg/g dry extract)[a]

Peak	Compound	Regression equation (µg/mL)	r^2	Linearity (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Precision		*J. glutinosa* (mg/g, dry extract)
							Inter-days (CV, %)	Intra-day (CV, %)	
	PHENOLIC COMPOUNDS								
	Phenolic acids								
1	3-*O*-Caffeoylquinic acid	$y = 2.063\times10^6x$	0.9991	1.875-120.0	0.3230	0.9798	8.641	3.486	0.9168 ± 0.07921
2	5-*O*-Caffeoylquinic acid	$y = 2.223\times10^6x$	0.9994	0.2716-198.0	0.03256	0.09867	6.375	1.035	12.32 ± 0.7855
3	4-*O*-Caffeoylquinic acid	$y = 2.116\times10^6x$	0.9994	1.781-114.0	0.07630	0.2312	7.764	2.876	0.6678 ± 0.05185
4	Caffeic acid	-	-	-	-	-	-	-	nq
5	1,3-di-*O*-caffeoylquinic acid	$y = 2.548\times10^6x$	0.9995	0.4453-114.0	0.05592	0.1695	10.13	1.518	0.5198 ± 0.05263
6	Isoferulic acid	$y = 3.921\times10^6x$	0.9994	0.4296-110.0	0.03260	0.09888	10.06	7.057	0.7829 ± 0.07876
7+8	3,4-di-*O*-caffeoylquinic acid + 3,5-di-*O*-caffeoylquinic acid[b]	$y = 2.455\times10^6x$	0.9973	0.4805-246.0	0.03829	0.1160	6.418	7.527	40.95 ± 2.628
9	1,5-di-*O*-caffeoylquinic acid	$y = 2.799\times10^6x$	0.9999	0.4375-112.0	0.1322	0.4006	9.888	2.682	24.73 ± 2.445
12	4,5-di-*O*-caffeoylquinic acid	$y = 2.537\times10^6x$	1.000	0.4531-116.0	0.2527	0.7659	10.88	6.630	23.14 ± 2.516
	Flavonoids								
10	Quercetin-3-*O*-galactoside	$y = 1.832\times10^6x$	0.9987	0.2908-212.0	0.08140	0.2467	8.219	1.695	15.16 ± 1.246
11	Quercetin-3-*O*-glucoside	$y = 1.878\times10^6x$	0.9992	0.07520-154.0	0.07796	0.2362	4.525	2.202	7.716 ± 0.3491
13	Quercetin	$y = 1.907\times10^6x$	0.9995	0.1387-124.0	0.08139	0.2466	8.149	1.189	5.015 ± 0.4087
14	Kaempferol	$y = 1.941\times10^6x$	0.9994	1.859-119.0	0.5339	1.6177	9.275	3.920	0.5244 ± 0.04864
15	Isorhamnetin	$y = 6.322\times10^5x$	0.9999	1.025-262.5	0.2252	0.6826	9.979	3.916	1.983 ± 0.2280
	Total phenolics								**134.4 ± 10.92**

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790 **Table 2. (continued)**

Peak	Compound	Regression equation (µg/mL)	r^2	Linearity (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Precision		*J. glutinosa* (mg/g, dry extract)
							Inter-days (CV, %)	Intra-day (CV, %)	
PIGMENTS									
	Carotenoids								
17	Lutein	$y = 2.492\times10^{2}x$	0.9992	0.07813-10.00	0.003416	0.01035	5.619	4.849	0.1477 ± 0.008299
23	β-carotene	$y = 6.713\times10^{2}x$	1.000	0.2743-66.67	0.03475	0.1053	9.129	5.548	0.01953 ± 0.001783
	Chlorophylls								
16	Chlorophyll *b*	$y = 9.459\times10^{1}x$	0.9998	0.5208-8.333	0.1027	0.3113	8.263	5.232	0.09917 ± 0.008194
	Total pigments								**0.2664 ± 0.01828**

[a] Results are expressed as the mean ± standard deviation of six determinations. LOD – limit of detection. LOQ – limit of quantification. [b] Quantified together as 3,5-dicaffeoylquinic acid. "nq" – not quantified