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## **Chemoprevention with a tea from hawthorn (*crataegus oxyacantha*) leaves and flowers attenuates colitis in rats by reducing inflammation and oxidative stress**

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## Journal Pre-proofs

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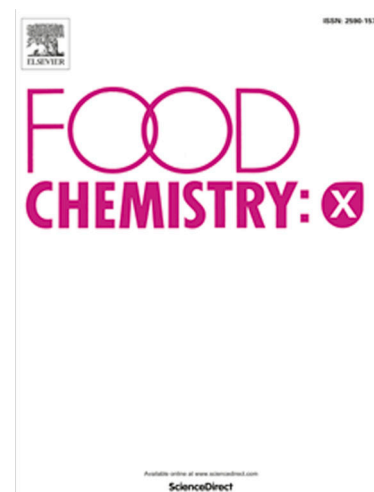
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*OXYACANTHA*) LEAVES AND FLOWERS ATTENUATES COLITIS IN RATS BY  
REDUCING INFLAMMATION AND OXIDATIVE STRESS

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28

## 29 ABSTRACT

30 The purpose of the study was to determine the effects of a tea from the leaves and flowers of  
31 *Crataegus oxyacantha* in rats with colitis. Colitis was induced by administration of 2,4,6-  
32 trinitrobenzene sulfonic acid. Hawthorn tea (HT) (100 mg/kg) was given via gavage for 21 days and  
33 the mesalamine drug (100 mg/kg) was administrated during the period of disease onset. HT was  
34 rich in total phenolic compounds (16.5%), flavonoids (1.8%), and proanthocyanidins (1.5%);  
35 vitexin-2-O-rhamnoside was the main compound detected. Mesalamine and the HT diminished the  
36 length of the lesions formed in the colon, in addition to reducing the levels of myeloperoxidase and  
37 interleukin-1 $\beta$ . Mesalamine was able to significantly reverse the body weight loss, while HT  
38 improved the activity of glutathione reductase and catalase. Histological scoring was not changed  
39 by the interventions, but it was highly correlated with the necrotic area. HT given at 100 mg/kg can  
40 be effective against colitis.

41

42 **Keywords:** *Crataegus oxyacantha*; polyphenol; vitexin-2-O-rhamnoside; inflammatory bowel  
43 diseases; colon.

44

## 45 ABBREVIATIONS

46 CAT - Catalase

47 CD - Crohn's disease

48 DAD - Diode array detection

49 DAI - Disease Activity Index

50 DSS - Dextran sodium sulfate

51 ELISA - Enzyme-linked immunosorbent assay

- 52 ESI - Electrospray ionization
- 53 FID - Flame ionization detector
- 54 FRAP - Ferric reducing antioxidant power
- 55 GC - Gas chromatograph
- 56 GPx - glutathione peroxidase
- 57 GR - Glutathione reductase
- 58 GSH - Glutathione
- 59 HT - Hawthorn tea
- 60 IBD - Inflammatory bowel disease
- 61 IL-1 $\beta$  - Interleukin-1beta
- 62 MDA - Malondialdehyde
- 63 MPO - Myeloperoxidase
- 64 MS - Mass spectrometry
- 65 ORAC - Oxygen-radical absorbing capacity
- 66 SCFA - Short-chain fatty acid
- 67 SOD - Superoxide dismutase
- 68 TFC - Total flavonoids content
- 69 TNBS - 2,4,6-trinitrobenzene sulfonic acid
- 70 TNF- $\alpha$  - Tumor necrosis factor-alpha
- 71 TPC - Total polyphenols content
- 72 TPOC - Total proanthocyanidin oligomers content
- 73 UC - Ulcerative colitis
- 74 UHPLC - Ultra-high-performance liquid chromatography

75

76 **1. INTRODUCTION**

77 in the last 20 years, there has been increasing *in vitro* and *in vivo* evidence of the beneficial  
78 health effects of functional foods, teas, and extracts from plants. Such alternative or complementary  
79 medicine has been suggested as a new tool against non-communicable diseases, which eventually  
80 may impact in the future for changes in prevention guidance and the treatment of debilitating  
81 chronic illness, such as cardiovascular diseases, inflammatory bowel diseases (IBDs), and cancer  
82 (Campos, 2019). However, more studies are needed in order to understand and deepen the effective  
83 dosages and mechanisms behind the biological action of natural bioactive compounds. Notably, the  
84 research on the utilization of the residues of plants, usually commercially discarded, such as stems,  
85 leaves, and flowers, is probably one of the strands that should be more investigated within the field  
86 of medicinal plants and herbs, given their great chemical and nutritional composition (Simpson et  
87 al., 2019).

88 Europe has a wide range of native plants spread through its territory, and one of the most  
89 common ones is hawthorn (*Crataegus spp.*), a tree belonging to the Rosaceae family (Chang et al.,  
90 2002). Hawthorn is commonly used as a hedging species in England and Denmark (Billing et al.,  
91 1974; Weber, 2010), and as a herbal remedy throughout the continent (Dahmer & Scott, 2010).  
92 Especially, the hawthorn extracts or teas from the leaves and flowers of *Crataegus oxyacantha* and  
93 *Crataegus monogyna* have been officially approved in Germany to treat patients with mild to  
94 moderate chronic congestive heart failure (Nathan, 1999) due to its high content in flavonoids and  
95 proanthocyanidins (Ngoc et al., 2019). It is known that flavonoids and proanthocyanidins can 1.  
96 decrease the inflammatory processes, 2. act by increasing the activity of enzymes that control the  
97 oxidation of the cell membrane (Panche et al., 2016; Rauf et al., 2019), and 3. possibly modulate the  
98 microbiome environment by being fermented by the microbiota (Cardona et al., 2013). Despite that,  
99 hawthorn has been mostly associated with beneficial effects in cardiovascular complications and  
100 hypertension (Dalli et al., 2011; Walker et al., 2006), while research on their effects in intestinal  
101 health and against gut diseases, like IBDs, for example, are lacking.

102 IBDs, mainly ulcerative colitis (UC) and Crohn's disease (CD), largely affect the  
103 gastrointestinal tract, producing a high-grade immune-associated inflammatory and oxidative  
104 process (Ungaro et al., 2017). Such diseases possess a high prevalence in Europe and are increasing  
105 at a high incidence rate since the '90s in newly industrialized countries, such as Brazil and Taiwan  
106 (Ng et al., 2017). Treatment options for IBDs are commonly not well accepted or tolerable for a  
107 great part of patients (Horne et al., 2009; Lasa et al., 2020; Pieper et al., 2009), suggesting the need  
108 for studies capable of proving new effective options. From our knowledge, four studies to date have  
109 attested the positive effects of hawthorn in IBDs; however, these investigations used the fruits, not  
110 the leaves or flowers, and distinct hawthorn species (Fujisawa et al., 2005; Guo et al., 2021; Liu et  
111 al., 2020; Malekinejad et al., 2013). Fujisawa et al. (2005) reported that the freeze-dried aqueous  
112 extract from the fruit of *Crataegi fructus* given in the drinking water prevented the inflammatory  
113 outcome and high mortality of chemically-induced models of colitis (Fujisawa et al., 2005). Liu et  
114 al. (2020) described that the freeze-dried hydroalcoholic extract from the fruit of *Crataegus*  
115 *pinnatifida*, rich in flavonoids, inhibited the secretion of proinflammatory cytokines and alleviated  
116 the increase of paracellular permeability in Caco-2 cells, therefore, indicating its protective effects  
117 against epithelial barrier dysfunction (Liu et al., 2020). Such initial studies indicate the potential of  
118 the hawthorn plant against IBDs.

119 No publication to date investigated the preventive effects of *Crataegus oxyacantha* or the  
120 tea from its flowering tops (leaves and flower buds) in broader aspects of IBDs, such as  
121 inflammation, oxidative stress, and microbiome health. Therefore, in order to understand if the  
122 effects of the residual parts of this plant are extended further than its most-known cardioprotective  
123 role, this study aimed to investigate the preventive implications of the administration of a  
124 polyphenol-rich dry tea from the *Crataegus oxyacantha* flowering tops in a model of IBD-like  
125 colitis induced in rats by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

126

## 127 2. MATERIAL AND METHODS

128 **2.1. Chemicals**

129 Aluminum chloride hexahydrate, n-butanol, Folin-Ciocalteu reagent, gallic acid (GA),  
130 quercetin (Q), and cyanidin chloride (CY) were purchased from Merck (Saint-Quentin Fallavier,  
131 France). *Crataegus spp.* extract standard (R1) was acquired from HWI Group (Rülzheim, Germany)  
132 and standardized at 29 mg of vitexin 2-O-rhamnoside per g of extract. Procyanidin A2, B2 and C1,  
133 epicatechin, cinnamtannin A2, and isoquercetin were purchased from Phytolab (Vestenbergsgreuth,  
134 Germany). 2,2'-Azobis(2-amidinopropane) dihydrochloride was purchased from Cayman Chemical  
135 (Ann Arbor, MI, USA), fluorescein was purchased from Synth (Diadema, SP, Brazil) and  
136 metaphosphoric acid was purchased from Vetec Quimica Fina Ltda. (Rio de Janeiro, RJ, Brazil).  
137 Protein Assay Dye Reagent Concentrate was acquired from Bio-Rad Laboratories (Hercules, CA,  
138 USA). Thiobarbituric acid was obtained from Merck KGaA (Darmstadt, Germany). Enzyme-linked  
139 immunosorbent assay (ELISA) kits were purchased from Peprotech (Rocky Hill, NJ, United States).  
140 1,1,3,3-tetraethoxypropane, 2,4,6-Tripyridyl-S-triazine, 5,5'-dithiobis-(2-nitrobenzoic acid), 6-  
141 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), bovine serum albumin, catalase  
142 (CAT), glutathione (GSH), myeloperoxidase (MPO), nitro blue tetrazolium chloride, oxidized  
143 glutathione, reduced nicotinamide-adenine dinucleotide phosphate, standards of short-chain fatty  
144 acids (SCFAs) (2-ethylbutyric acid, acetic acid, butyric acid, and propionic acid), sodium dodecyl  
145 sulfate and TNBS were all obtained from Sigma Aldrich (St. Louis, MO, USA).

146

147 **2.2. Hawthorn tea production and chemical characterization**

148 Dry hawthorn flowering tops (*Crataegus oxyacantha*) (leaves and flower buds) were  
149 purchased from France Herboristerie (Lot number 55849, Noidans-Lès-Vesoul, France). The plants  
150 were previously grounded using an Ika grinder (Ika-Werke GmbH, Model MF10 basic, Staufen,  
151 Germany) and a “1 mm” grid. The material size distribution was determined by dry laser  
152 granulometry.



153 The tea was produced according to NGOC et al. (NGOC et al., 2019). A sample of 50 g was  
154 infused in 1 L of boiling water using a 'French press' Bodum® under 500 rpm magnetic stirring.  
155 After 30 min, the herbal tea solution was filtered first with the Bodum® cover to remove the largest  
156 particles, then with a Whatman filter paper placed in a Büchner funnel using a vacuum pump to  
157 remove any residue of the solid plant. Finally, the herbal tea solution was concentrated using a  
158 rotary evaporator and later freeze-dried (Cryotec Model CRIOS-80, Saint-Gély-du-Fesc, France) to  
159 obtain the dry hawthorn tea (HT). The same experiment was repeated three times to get a total of 30  
160 g. Lyophilized tea was stored at 4°C (Ngoc et al., 2019).

161 Total polyphenols (TPC) (mg of gallic acid equivalent/g dry weight), total flavonoids (TFC)  
162 (mg of quercetin equivalent/g dry weight), and total proanthocyanidin oligomers (TPOC) (mg of  
163 cyanidin equivalent/g dry weight) contents were determined according to the Folin-Ciocalteu  
164 method (Singleton & Rossi, 1965), the aluminum chloride method (Lamaison & Carnat, 1990) and  
165 the HCl/n-butanol assay (Porter et al., 1985), respectively. Analyses were performed in triplicate.

166 For the quantification and identification of specific phenolic compounds, an ultra-high-  
167 performance liquid chromatography associated with diode array detection (UHPLC-DAD) and  
168 coupled with electrospray ionization and mass spectrometry (UHPLC-ESI-MS), respectively, were  
169 utilized. First, 20 mg of the dry HT was dissolved in 1 mL of water and vortexed for 2 min. The  
170 resulting solution was diluted five times with water and vortexed for another 2 min prior to the  
171 analysis.

172 The UHPLC-DAD system consisted of a Thermo Scientific™ Dionex™ UltiMate™ 3000  
173 BioRS equipped with a WPS-3000TBRS autosampler and a TCC-3000RS column compartment set  
174 at 35°C (ThermoFisher Scientific, Waltham MA, USA). The system was operated using  
175 Chromeleon 7 software. A Luna® Omega polar C18 column (1.6 µm, 100 × 2.1 mm) combined  
176 with a security guard ultra-cartridge was used (Phenomenex Inc., Torrance CA, USA). A binary  
177 solvent system was utilized, consisting of water/formic acid (1%, v/v) as solvent A and  
178 acetonitrile/formic acid (1%, v/v) as solvent B. The gradient program started with 5% B, then B was

179 increased to 100% in 30 min with a convex increase (curve 5 in Chromeleon 7). The flow rate of  
180 the mobile phase was 0.4 mL/min, and the injection volume was 4  $\mu$ L. The peaks were monitored at  
181 273 nm. The UV-Vis spectra of the different compounds were recorded between 200 and 550 nm  
182 using the diode array detector.

183 The UHPLC-ESI-MS analysis was performed using a Synapt G2-S (Waters Corp., Milford  
184 MA, USA) equipped with an ESI. The column, injection volume, flow rate, and gradient program  
185 were the same as for UHPLC-DAD. A positive mode was used. The capillary voltage was set to 3  
186 kV, the cone voltage was set to 30 V, and the extract or voltage was set to 3 V. The source  
187 temperature was 100°C, and the desolvation temperature was 450°C. MS spectra were obtained by  
188 scanning ions between 100 and 1500 m/z. The system was operated using MassLynx 4.1 software.

189 The quantification of vitexin-2-O-rhamnoside, a common and abundant flavonoid found in  
190 hawthorn species (Alirezalu et al., 2018; Kumar et al., 2012), was determined by external  
191 calibration, using a commercially available standard. All other components identified in the HT  
192 were expressed as peak area ratio from the chromatogram, since the standards were not available.

193

## 194 **2.3. Animal experimentation**

### 195 **2.3.1. Ethics, diet, and conditions**

196 The study is in accordance with the ARRIVE guidelines and followed the guide for the care  
197 and use of laboratory animals of the National Institutes of Health (NIH Publications No. 8023,  
198 revised 1978), and the Brazilian National Council for Animal Experiments Control - CONCEA.  
199 The Ethics Committee on the Use of Animals of the University of Campinas approved the  
200 experimental protocol (number 5042-1/2018, UNICAMP).

201 Male Wistar rats (n=29) were obtained from the Multidisciplinary Center for Biological  
202 Research on Laboratory Animal Science at UNICAMP and allocated in an experimentation room  
203 with the temperature at controlled levels (20-22°C). Rats were submitted to a standard daily 12 h:12  
204 h light-dark cycle. During the acclimatization and experimental periods, rats received a commercial

205 pelleted diet (NUVITAL CR-1, NUVITAL) and water *ad libitum*. Animals entered the experimentation  
206 room at four weeks of age and were submitted to the procedures starting at seven weeks of age.

207

### 208 2.3.2. Intervention, colitis induction, and clinical evaluation

209 A total of 29 Wistar rats were divided in five experimental groups: *control-C* (n=5), *control*  
210 *colitis-CC* (n=6), *hawthorn tea-H* (n=5), *hawthorn tea colitis-HC* (n=6) and *mesalamine colitis-MC*  
211 (n=7). Groups with colitis had more animals since death may happen in the TNBS model (Antoniou  
212 et al., 2016). Dry HT was diluted in distilled water and given preventively for 14 days at a dosage  
213 of 100 mg/kg of body weight via gavage. The dosage chosen was based on the majority of studies  
214 found in the literature (Elango et al., 2009; Elango & Devaraj, 2010; Hatipoğlu et al., 2015). The  
215 intervention with HT (groups *H* and *HC*) continued until the end of the experiment, including the  
216 period after colitis induction (when applicable). Other groups (*C*, *CC*, *MC*) received distilled water  
217 via gavage instead.

218 Acute colitis was induced on the 14<sup>th</sup> day using TNBS (10 mg) dissolved in 250 µL of 50%  
219 ethanol (v/v) (da Silva-Maia et al., 2019) and administrated via rectal in rats previously sedated  
220 intraperitoneally with ketamine (75 mg/kg of body weight) and xylazine (10 mg/kg of body  
221 weight). Groups without colitis induction (*C*, *H*) received saline solution (0.9%) instead of TNBS.

222 Mesalamine, a drug commonly used to treat IBDs (Lacucci et al., 2010), was diluted in  
223 distilled water and given via gavage at a dosage of 100 mg/kg/day (da Silva-Maia et al., 2019).  
224 Mesalamine was administrated for seven days, from the day of the induction until the end of the  
225 experiment.

226 The dietary intake (g) and body weight (g) were measured during the whole experiment.  
227 Additionally, for the period of colitis, the Disease Activity Index (DAI) was assessed (days 14, 15,  
228 17, 19, and 20), according to described by Gommeaux et al. (2007). The DAI evaluates the body  
229 weight loss, anal bleeding, and stool consistency (Gommeaux et al., 2007). Photographs of the rat's  
230 stool and anus were taken to analyze anal bleeding and stool consistency. A blinded researcher to

231 animals' identification and with experience in IBDS, did the evaluations. A photographic  
232 representation of the scores of bleeding and stool consistency from this study was created similarly  
233 to Nascimento, Lima, et al. (2020) to showcase the differences between the score's degrees. A  
234 Nikon® SLR D3100 digital camera with 14.2-megapixel resolution and an 18-55 mm lens was  
235 utilized.

236

### 237 **2.3.3. Euthanasia and tissue measurements**

238 Euthanasia was performed on day 21 by exsanguination through cardiac puncture.  
239 Intraperitoneal administration of ketamine (300 mg/kg of body weight) and xylazine (30 mg/kg of  
240 body weight) were used to anesthetize the animals.

241 Colon was dissected, cleaned with saline solution, weighted (g), and measured with a ruler  
242 (cm). The colon weight/length (g/cm) ratio was analyzed. Brownish necrotic lesions are common  
243 features of TNBS-induced colitis (De Almeida et al., 2015; Paiotti et al., 2013); therefore, their  
244 width (cm), length (cm), and estimated area (width x length, cm<sup>2</sup>) were measured for this study.  
245 Additionally, the relation between the larger necrotic lesion length (cm) and the colon length (cm)  
246 was evaluated as an indicator of the colon proportion (%) affected by the disease. A small portion of  
247 the distal colon was separated for histology. The remaining, composed of proximal, middle, and  
248 distal colon, and rectum, were mixed and preserved at -80°C for further analyses (inflammation and  
249 oxidative stress).

250 Blood was centrifuged, and the serum was collected and stored at -80°C for posterior  
251 antioxidant analyses. Feces from the cecum and colon were collected and kept at -80°C for the  
252 analysis of the concentration of SCFAs. The liver, spleen, and kidney were weighted (g). The  
253 carcasses of the animals and unused tissues or organs were discarded as biological materials.

254

## 255 **2.4. Histopathology**

256 A sample of the distal colon was disposed in a small piece of paper and preserved in  
257 formaldehyde 4% (v/v) until histological processing. Briefly, samples were dehydrated with  
258 increasing ethanol concentrations, included in paraffin blocks, and subjected to sectioning (4  $\mu\text{m}$ )  
259 follow by insertion in slides. Slides were standardly stained with hematoxylin and eosin and  
260 analyzed by a specialist, blind to the identification of the groups, according to a score modified  
261 from Dieleman et al. (1998) (**Table 1**). Photos were taken at 10x/0.25 magnification, 6.31x/0.25  
262 AxioCam ICc5 (Zeiss, Germany).

263

## 264 **2.5. Antioxidant capacity of the serum**

265 The total antioxidant capacity of the serum was evaluated by two methods, the ferric-  
266 reducing antioxidant power (FRAP) and the oxygen-radical absorbing capacity (ORAC). Trolox  
267 was utilized as a standard for both analyses. Before the tests, samples were submitted to treatment  
268 with metaphosphoric acid in order to precipitate proteins and extract the antioxidant substances  
269 (Leite et al., 2011).

270 For the FRAP analysis, samples were added with a solution composed of 2,4,6-tripyridyl-S-  
271 triazine, ferric chloride, and acetate buffer at proportions of 1:1:10, respectively. Samples were  
272 included in a microplate and incubated for 30 min at 37°C. The absorbance was read at 595 nm  
273 using an Epoch™ spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA), which was  
274 utilized for all the applicable analyses. The results were expressed in  $\mu\text{mol}$  of Trolox equivalent/mL  
275 of serum (Benzie & Strain, 1996).

276 For the ORAC analysis, in a microplate, samples were added with fluorescein and incubated  
277 for 10 min at 37°C. After that, 2,2'-azobis(2-amidinopropane) dihydrochloride was added, and the  
278 fluorescence was read for 80 min with filters set at 520 nm for emission and 485 nm for excitation.  
279 The area under the curve was calculated, and the results were expressed in  $\mu\text{mol}$  of Trolox  
280 equivalent/mL of serum (Ou et al., 2013).

281

282 **2.6. Inflammation and oxidative stress analyses**

283 Colonic samples were homogenized in a potassium phosphate buffer (75 mM, pH 7.4) and  
284 centrifuged at 10,000 rpm for 30 min. The supernatant was collected and subjected to protein  
285 quantification (mg of bovine serum albumin/mL) by the Bradford assay (Bradford, 1976).  
286 Supernatants were utilized for the determination of MPO and cytokines concentration, and the  
287 oxidative stress analyses.

288 The concentration of MPO (U/g protein), tumor necrosis factor-alpha (TNF- $\alpha$ ), and  
289 interleukin-1beta (IL-1 $\beta$ ) (ng/g of protein or mL of serum) were determined as indicators of  
290 inflammation. MPO was determined by mixing the diluted homogenates (1:4) or a standard with a  
291 cocktail of O-dianisidine dihydrochloride and hydrogen peroxide. MPO was utilized as the  
292 standard. Absorbance was read every min for 10 min at 460 nm, and the area under the curve was  
293 calculated (Winterbourn et al., 1975). The concentrations of the proinflammatory cytokines TNF- $\alpha$   
294 and IL-1 $\beta$  in colon homogenates and serum were determined by ELISA, following the protocols of  
295 commercial kits.

296 The activity or concentration of CAT (U/g of protein), glutathione reductase (GR)  
297 (consumed NADPH/min/g of protein), GSH (nmol of GSH/mg of protein), malondialdehyde  
298 (MDA) (nmol of MDA/g of protein), and superoxide dismutase (SOD) (SOD/g of protein) were  
299 determined as indicators more closely related to oxidative stress.

300 The CAT assay was realized by mixing first the diluted homogenates (1:2) with hydrogen  
301 peroxide and incubating for two min at 37°C. After that, ammonium metavanadate was included,  
302 and the absorbance was read after 10 min at 452 nm. CAT was utilized as a standard. CAT activity  
303 was determined by a logarithmic formula, adapted from a recent methodological approach (Hadwan  
304 & Ali, 2018).

305 For the GR analysis, homogenates (diluted at 1:1 or not diluted, depending on the sample)  
306 were added with a cocktail containing reduced nicotinamide-adenine dinucleotide phosphate,

307 oxidized glutathione, and ethylenediaminetetraacetic acid. The reading was realized at 340 nm per  
308 min for 10 min and the area under the curve was calculated (Carlberg & Mannervik, 1985).

309 GSH was determined by first mixing a tris reagent plus ethylenediaminetetraacetic acid  
310 buffer with diluted homogenates (1:4) or a standard and read at 421 nm. This step was followed by  
311 the inclusion of 5,5'-dithiobis-(2-nitrobenzoic acid) and a 15 min incubation period. The second  
312 reading was also done at 421 nm. GSH was utilized as the standard. The calculation was realized by  
313 subtracting the first reading data from the second one, followed by linear regression (Yoshikawa et  
314 al., 1993).

315 The concentration of MDA was determined by the thiobarbituric acid reactive substances  
316 (TBARS) method. Briefly, samples or a standard were mixed with sodium dodecyl sulfate and a  
317 solution containing thiobarbituric acid, sodium hydroxide, and acetic acid. This mixture was left for  
318 60 min in a boiling water bath and then cooled for 10 min. Samples were centrifuged at 10,000 rpm  
319 for 10 min at low temperature (4°C). The absorbance of the supernatant was read at 532 nm. A  
320 standard curve was made using 1,1,3,3-tetraethoxypropane, and the results were expressed as nmol  
321 MDA/g protein (Ohkawa et al., 1979).

322 SOD was quantified by mixing the diluted homogenates (1:4) with a cocktail composed of  
323 hypoxanthine, xanthine oxidase, and nitro blue tetrazolium chloride. Absorbance was read every  
324 min for 10 min at 560 nm, and the area under the curve was calculated (Winterbourn et al., 1975).

325

## 326 **2.7. Short-chain fatty acids concentration**

327 Feces sample preparation followed the protocol described by Zhao et al. (2006), with  
328 modifications. Approximately 300 mg of stool was diluted in distilled water (1:6) and mixed with  
329 HCl for adjustment in pH 2. Afterward, the samples were centrifuged at 3000 rpm for 60 min. The  
330 supernatant was collected and mixed with the internal standard 2-ethyl-butyric acid. Once prepared,  
331 the samples were injected into a gas chromatograph coupled to a flame ionization detector. The  
332 samples were injected with an auto-injector brand Shimadzu, model Ai20, in a GC-FID Shimadzu,

333 model GC-2010 plus, equipped with a capillary column of fused silica NUKOI (30 m x 0.25 mm,  
334 inside diameter x 0.25  $\mu$ m). The chromatographic conditions were as follow: injector at 200°C  
335 operating in split mode 1:5 for 1.0 min; helium carrier gas at 1 mL/min; oven temperature ramp  
336 starting at 100°C, with an increase of 8°C/min to 190°C, remaining at this temperature for 3.25 min;  
337 and detector at 200°C. The co-injection of authentic standards identified the analytes. All  
338 chromatographic analyzes were performed in triplicate (Zhao et al., 2006).

339

## 340 **2.8. Statistical analysis and Pearson's correlation**

341 Results are presented as mean  $\pm$  standard deviation (SD) for data from the chemical  
342 characterization and as mean  $\pm$  standard error of the mean (SEM) for data from the biological  
343 analyses.

344 For data from the biological analysis, first, outliers were discarded after realizing the Grubbs  
345 test (5%). Then, One-way ANOVA followed by Tukey (parametric data) was utilized when making  
346 comparisons among all experimental groups. Two-way ANOVA followed by Tukey (parametric  
347 data) was utilized for analyses over time (body weight, DAI). Data between groups were considered  
348 statistically different when  $p < 0.05$ .

349 Data were submitted to Pearson's correlation and included in a correlation matrix. The  
350 correlations were only considered parameters previously attested to be significantly altered by the  
351 induction of colitis or the intervention with the HT ( $p < 0.05$ ). Groups without colitis induction (C,  
352 H) were not included, as they could bias the results. Pearson's  $r$  placed between 0.70 and 1  
353 (positive) or -0.70 and -1 (negative) were classified as highly correlated (Hinkle et al., 2003). A  
354 heatmap was created to represent the correlation matrix.

355 Analyses were performed using GraphPad Prism.

356

## 357 **3. RESULTS AND DISCUSSION**

### 358 **3.1. Tea characterization and dosage of compounds administrated to rats**



359 The extraction yield was about 20% in mass, meaning that 200 mg of HT was obtained from  
360 1 g of the dry ground plant. The contents of TPC, TFC and TPOC obtained from HT were  $165.55 \pm$   
361  $2.20$  mg of gallic acid equivalent/g of dry tea,  $18.8 \pm 0.70$  mg of quercetin equivalent/g of dry tea,  
362 and  $15.35 \pm 0.60$  mg of cyanidin equivalent/g of dry tea, respectively. The levels found from the  
363 three analyses are in accordance with recent studies using similar plant species and portions (Issaadi  
364 et al., 2020; Ngoc et al., 2019). By utilizing only water for the extraction of the ground plant, the  
365 results of TPC ( $33.11$  mg/g of dry plant) shown in the present study are comparable or even higher  
366 than less green extracts utilized by the literature (Edwards et al., 2012). For example, in a study by  
367 Alirezalu et al. (2018), the TPC of different *Crataegus* species (flowers and leaves), extracted with  
368 methanol/water (80%, v/v), ranged between 20 to 50 mg/g. Also, in a study by Keser et al. (2014),  
369 the polyphenol content of the aqueous extract of *Crataegus monogyna* leaves and flowers was  
370 found to be higher than the extract with 100% ethanol, while also possessing a high antioxidant  
371 activity (Keser et al., 2014). Such findings suggest that the use of water for the extraction of  
372 hawthorn can be equally important to other solvents, as far as the dry plant is ground ( $< 1$  mm).

373 The main compounds found in the HT were flavonoids, like chlorogenic acid, vitexin 2-O-  
374 rhamnoside, pinnatifinoside A, hyperoside, and apigenin-C-hexoside, as detected by the UHPLC  
375 retention time (**Table 2**) and peak (**Fig. 1A**) profiles. Especially, vitexin 2-O-rhamnoside presented  
376 the highest relative peak area (**Fig. 1B**), as later quantified. The content of this flavonoid by  
377 UHPLC was determined to be  $3.53 \pm 0.036$  mg/g of dry plant or  $17.66 \pm 0.18$  mg/g of dry HT.  
378 Vitexin 2-O-rhamnoside is the main flavonoid found in hawthorn species, with content reaching up  
379 to 6.6 mg/g of dry plant (Martino et al., 2008) or 26 mg/g of the tea (i.e., 5.2 mg/g of dry tea) (Ngoc  
380 et al., 2019). Studies have shown the capacity of vitexin 2-O-rhamnoside against induced-oxidative  
381 stress damage (Wei et al., 2014) and endothelial injury (Zhu et al., 2006), making it also a probable  
382 candidate for inflammatory intestinal conditions. However, such flavonoid has poor oral  
383 bioavailability in rats (less than 5%) (Gao et al., 2016), which could indicate its biotransformation

384 by the microbiota in similarity to other prebiotic-like flavonoids (e.g., anthocyanins) (Kawabata et  
385 al., 2019), but studies are needed to investigate such assumption.

386 Considering the results from the chemical characterization of the hawthorn plant, rats  
387 received 16.5 mg of total phenolic compounds/kg of body weight and 1.76 mg of vitexin 2-O-  
388 rhamnoside/kg of body weight daily during 14 days for prevention and the following 7 days while  
389 with colitis.

390

### 391 3.2. Macroscopic and microscopic parameters

392 Colitis was successfully induced in rats, as seen by an increased body weight loss (**Fig. 2A**)  
393 and high DAI after one day from the colitis induction ( $p < 0.01$ ) (**Fig. 2B**). In general, animals with  
394 colitis presented softening of the stools and slight bleeding, as shown by the photographic  
395 representation of their respective scores in **Figs. 3** and **4**, respectively. As expected, the treatment  
396 with 100 mg/kg of mesalamine recovered the animals' body weight at the end of the experiment  
397 ( $p < 0.001$ ) compared to the *CC* group. Mesalamine is commonly utilized for IBDs, as it can block  
398 cyclooxygenases, interleukins, and TNF- $\alpha$ , therefore controlling the inflammatory process and  
399 contributing to preventing common intestinal symptoms and body weight loss (Lacucci et al.,  
400 2010). HT could not significantly reverse the body weight loss ( $p = 0.0560$ ) in rats with colitis  
401 (group *HC*), but animals tended to return to their initial body weight before colitis induction (**Fig.**  
402 **2A**).

403 Only the rats in the *CC* group presented a high colon weight/length ratio ( $p < 0.05$ ) compared  
404 to the *C* group (**Fig. 2C**), potentially indicating the anti-inflammatory effects of the interventions  
405 with mesalamine and HT. The occurrence of necrotic lesions was evident in all rats that received  
406 TNBS (**Fig. 2D**). Both mesalamine and HT significantly decreased the length ( $p < 0.001$ ) and the  
407 area ( $p < 0.01$ ) of the most extensive brownish necrotic lesion, as well as the proportion of the colon  
408 affected by TNBS ( $p < 0.05$ ) compared to the *CC* group. No differences were found between the *MC*  
409 and *HC* groups regarding such parameters (**Table 3**).

410 The anti-necrotic potential of extracts from hawthorn is still poorly documented (Fujisawa et  
411 al., 2005; Zapatero, 1999), but evidence indicates that flavonoids and phenolic acids (e.g.,  
412 chlorogenic acid), which were identified in the HT of our study (**Fig. 1, Table 2**), possess such  
413 properties. For example, chlorogenic acid, a polyphenol found in coffee beans, apple, and hawthorn  
414 species (Alirezalu et al., 2018), and the vitexin-rich plant *Tragopogon graminifolius*, are effective in  
415 reducing the typical macroscopic and necrotic lesions of TNBS-induced colitis (Farzaei et al., 2015;  
416 Zatorski et al., 2015; Zhou et al., 2016). Although necrosis is not a typical documented finding of  
417 patients with IBDs, it can work as a parameter to measure the possible anti-inflammatory and pro-  
418 homeostatic effects of natural products.

419 Although alterations in the liver, spleen, and kidney are described in few studies that use the  
420 model of TNBS-induced colitis (Jang et al., 2018; Patel & Trivedi, 2017; Zhi et al., 2017), in the  
421 present study, no differences were found between the experimental groups regarding the  
422 macroscopic aspect and weight of such organs (results not shown).

423 As far as the histological analysis, TNBS promoted extensive and severe ulceration in the  
424 epithelium, with loss of crypts, and a high inflammatory income in the mucosa and submucosa (**Fig.**  
425 **5**). Results from the histological grading significantly shown this for all colitis groups ( $p < 0.05$ ).  
426 Mesalamine ( $p = 0.103$ ) and HT ( $p = 0.318$ ) did not statistically decrease the histological score (**Table**  
427 **3**), which could probably be related to two possibilities: 1. as expected for the TNBS model  
428 (Antoniou et al., 2016), the microscopic severity was extremely high, especially in the area selected  
429 for histology (distal colon), making it difficult for a clear observation of the benefits of the  
430 treatments; 2. the HT only had a mild preventive effect that could not be translated in the  
431 microscopic analysis. The next step would be to perform a study with other colitis model to  
432 understand if HT could exert preventive effects in the histology of the damaged intestinal mucosa.  
433 Cinthia B.B. Cazarin et al. (2014), for example, by using the *Passiflora edulis* peel (rich in  
434 polyphenols and dietary fibers) could not find statistical significance in the microscopic score of  
435 rats with TNBS-induced colitis, but when they utilized the dextran sodium sulfate (DSS) model, a

436 significant reduction in the histological damage in the colon was observed (Cintia Bau Beum  
437 Cazarin et al., 2016). The DSS model results in less severe inflammation and more superficial  
438 ulcers in rats when compared to the TNBS acute model (Catana et al., 2018), which typically  
439 triggers extensive transmural inflammation (Antoniou et al., 2016). According to a recent review  
440 (Nascimento, Machado, et al., 2020), the DSS model best mimics the inflammation of UC, while  
441 the TNBS model, the inflammation of CD. In studies with colitis using products from hawthorn  
442 berries, the histological damage is improved, however, the models associated with the results are  
443 acetic acid and DSS, and not TNBS (Fujisawa et al., 2005; Guo et al., 2021; Malekinejad et al.,  
444 2013).

445

### 446 **3.3. Serum antioxidant capacity**

447 No statistical differences were found between all groups regarding the results from the  
448 FRAP and ORAC methods, meaning that the TNBS model did not worsen the systemic antioxidant  
449 capacity; neither did the intervention with HT increase it (**Table 4**). In a study by Luana H. Maurer  
450 et al. (2020), similarly, ORAC and FRAP data were not changed by the TNBS-colitis model or by  
451 the intervention with a rich source of polyphenols (Luana H. Maurer et al., 2020). Since our study  
452 utilized an acute colitis model, we understand that the serum was not deeply affected. Maybe, a  
453 different dosage of HT may be necessary to achieve significance for such analyses.

454

### 455 **3.4. Concentration or activity of inflammatory proteins and oxidative metabolism enzymes**

456 As expected, MPO and IL-1 $\beta$  levels were significantly increased by the inflammatory  
457 process in the colon ( $p < 0.01$ ). Both mesalamine and HT decreased the concentrations of MPO and  
458 IL-1 $\beta$  ( $p < 0.05$ ) (**Table 4**). Similarly, Liu et al. (2020) and Malekinejad et al. (2013), by using  
459 flavonoid-rich extracts from hawthorn berries, also found reduced levels of IL-1 $\beta$  and MPO,  
460 respectively, in their pre-clinical studies with colitis. Concentrations of IL-1 $\beta$  in serum and TNF- $\alpha$   
461 in colon and serum were not changed in our study (**Table 4**).

462 Besides the common pathway of release of the IL-1 $\beta$  by macrophages (caspase-1 activation  
463 by inflammasome), neutrophil proteases can also cleave the precursor of this cytokine into its  
464 biologically active forms (Guma et al., 2009), following tissue injury and cell necrosis. Also, to help  
465 create an IL-1 $\beta$ -dependent inflammatory response (Lopez-Castejon & Brough, 2011), neutrophils  
466 can express MPO, a local mediator of tissue damage and inflammation ignition (Aratani, 2018).  
467 Patients with IBDs possess high serum and fecal levels of IL-1 $\beta$  and MPO (Peterson et al., 2007;  
468 Vasilyeva et al., 2016), and the typical inflammation of IBDs is largely attributed to the  
469 accumulation (UC) or impaired function (CD) of neutrophils (Wéra et al., 2016). In the present  
470 study, HT was able to decrease the levels of IL-1 $\beta$  and MPO in the colon, indicating its potential to  
471 block or regulate intestinal inflammations associated with the accumulation and activation of  
472 intestinal mucosal neutrophils (Therrien et al., 2019).

473 Regarding the results of oxidative metabolism enzymes, CAT activity was increased by the  
474 colitis model ( $p < 0.01$ ). Experimental studies with mice supplemented with strains of genetically  
475 modified bacteria found that elevated CAT activity in a TNBS-induced CD murine model is linked  
476 to a decreased likelihood of worsening the disease and developing colon cancer (LeBlanc et al.,  
477 2011). Observing **Table 4**, only the colitis group treated with the HT (group *HC*) showed a  
478 normalization in CAT levels ( $p < 0.01$ ), demonstrating a superior effect of a natural alternative  
479 intervention in comparison with a drug. Additionally, the colitis group receiving HT highly  
480 increased the levels of GR in comparison to *CC* and *MC* ( $p < 0.001$ ) (**Table 4**). This increase  
481 possibly happened in response to the production of glutathione peroxidase (GPx), an antioxidant  
482 enzyme that works in conjunction with CAT by transforming hydrogen peroxide ( $H_2O_2$ ) into water  
483 ( $H_2O$ ), thus decreasing oxidative stress caused by inflammation (Ighodaro & Akinloye, 2018).  
484 Briefly, in order for the peroxisome organelle to obtain GPx, it is necessary to convert reduced  
485 glutathione (GSH) into its oxidized form (GSSH). For the regeneration of GSH (glutathione cycle),  
486 the presence of GR is necessary (Moura et al., 2015).

487 Both CAT and GR are also known for their role in lipid peroxidation. CAT acts as a first-  
488 line defense against the formation of free radicals, while GR is responsible for the regeneration of  
489 GSH, the body master's antioxidant (Fagan & Palfey, 2010; Ighodaro & Akinloye, 2018; Pérez et  
490 al., 2017). According to recent studies, GR can be increased in rats with improved TNBS-induced  
491 colitis, and that received rich sources of flavonoids (jaboticaba peel and grape peel residues) (da  
492 Silva-Maia et al., 2019; Luana Haselein Maurer et al., 2019). This scenario suggests the importance  
493 of GR restoration to ameliorate intestinal conditions. On the other side, despite the well-known  
494 regulating role of CAT, in the present study, its activity was increased after colitis induction. This  
495 modification is probably due to adaptive or defective mechanisms, as this enzyme has also been  
496 found highly expressed in neutrophils from the intestinal mucosa of patients with IBDs (Kruidenier  
497 et al., 2003). Therefore, besides acting in the MPO-IL-1 $\beta$  axis, HT could also positively interfere in  
498 the mechanics of CAT and GR, first by avoiding the excessive or defective action of CAT, then by  
499 highly increasing the levels of GR to control the propagation of reactive oxygen species and restore  
500 redox balance.

501 The levels of SOD, TBARS, and GSH in the colon were not changed either by the colitis  
502 model or the interventions (**Table 4**).

503

### 504 **3.5. Concentration of short-chain fatty acids**

505 The extract of the *Crataegus oxyacantha* leaves is reported to increase the growth of  
506 *Bifidobacterium* and *Lactobacillus* species and the concentration of acetic acid, according to an *in*  
507 *vitro* study using skim milk as a fermentable matrix (Khaleel & Haddadin, 2013). Another study  
508 showed that flavonoids, including chlorogenic acid, which is abundant in *Crataegus oxyacantha*,  
509 slightly increased the counts of *Bifidobacterium spp.* and the concentration of SCFAs *in vitro*  
510 (Parkar et al., 2013). However, in the present study, the concentration of SCFAs (total, acetic,  
511 butyric, propionic) in feces from both cecum and colon of rats was not altered by the HT.

512 Additionally, these compounds were not modulated by the TNBS-colitis induction model (data not

513 snown). Perhaps, the polyphenols dosage in this study may not be sufficient to increase the levels of  
514 SCFAs, or the treatment period was short. Since our study used oral gavage to inoculate the  
515 proposed treatment, we decided to not extend its duration. Gavage, especially if done for a  
516 prolonged period and repeatedly, may cause reflux, irritation, and inflammation of the esophagus  
517 (Damsch et al., 2011; Kinder et al., 2014). Longer treatment periods using diets enriched with  
518 hawthorn products may encounter better results as far as SCFAs levels.

519 A recent investigation using a treatment with hawthorn for chemically-induced colitis found  
520 microbiota modulation and increased levels of SCFAs *in vivo*. However, a diversity of aspects were  
521 different from our study, which include: the animals used (C57BL/6J mice), the colitis model  
522 (DSS), the fraction and species of the hawthorn plant (fruit of *Crataegus pinnatifida*), and  
523 especially, the treatment applied and its period (an extracted polysaccharide for 42 days) (Guo et al.,  
524 2021). Such promising evidence indicates that the microbiome-related aspects of the therapy with  
525 the hawthorn plant are worthy of investigation and need further experiments to confirm its potential  
526 *in vivo*.

527

### 528 3.6. Correlation matrix

529 Colon weight/length ratio, the area of the necrotic lesions, and IL-1 $\beta$  levels presented the  
530 most numerous and diverse correlations. On the contrary, the DAI at its peak day had overall weak  
531 correlations with all other parameters (**Fig. 6**).

532 The colon weight/length ratio, which is regarded as an inflammatory index (Sánchez-  
533 Fidalgo et al., 2010), was highly correlated with almost all the lesion parameters and the levels of  
534 IL-1 $\beta$ . On the other side, this cytokine was positively correlated with MPO levels ( $r=0.846$ ),  
535 probably due to mechanisms previously mentioned, associated with neutrophil infiltration and the  
536 release of inflammatory markers in the colonic mucosa. MPO also showed a high negative  
537 correlation with body weight alteration ( $r=-0.708$ ). In a trial with patients with UC, MPO and IL-1 $\beta$   
538 had good correlations with the histological, endoscopic, and clinical severity of the disease

539 (Peterson et al., 2007). This scenario suggests the possibility of measuring such markers to improve  
540 prognostic in IBDs, in addition to targeting compounds capable of specifically blocking them, such  
541 as the ones found in the HT.

542 There was also a negative correlation ( $r=-0.732$ ) between the levels of CAT and GR. As  
543 previously mentioned, this data shows that HT is mechanistically acting through an oxidative stress  
544 pathway, mainly by affecting in opposite ways GR and CAT in order to prevent the spread of free  
545 radicals and consequently oxidative stress.

546 Although the interventions did not reduce the histological score, there was a high positive  
547 correlation of such parameter with the necrotic lesion area ( $r=0.708$ ), which was diminished by the  
548 HT.

549

#### 550 4. CONCLUSIONS

551 Animal studies directed towards IBDs lack analysis or do not present an adequate  
552 experimental model (e.g., acetic acid-induced colitis). This study is, to the best of our knowledge,  
553 the first successful investigation of the effects of *Crataegus oxyacantha* in IBDs. A HT prepared  
554 from the flowering tops (leaves and flowers), a source of polyphenols, effectively decreases  
555 inflammatory mediators (IL-1 $\beta$ , MPO) and regulates oxidative stress in TNBS-induced colitis.  
556 Additionally, administration of HT via oral gavage does not cause any harm in healthy rats. Such  
557 findings indicate that the hawthorn plant may serve as an alternative or complementary natural  
558 remedy that extends beyond its well-known cardiovascular protective and anti-hypertensive roles.

559

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568

## 569 **DECLARATION OF INTEREST**

570 None.

571

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TABLE 1. HISTOLOGICAL GRADING OF COLITIS.

Score	Leukocyte infiltration	Inflammation extent	Crypt damage
0	None	None	None
1	Discrete	Mucosa	1/3 damaged (basal)
2	Moderate	Mucosa and submucosa	2/3 damaged (basal)
3	Severe	Transmural	Only the surface of the epithelium is intact
4	-	-	Loss of crypt and epithelium

Area involved in percentage (score): 1–25 (1), 26–50 (2), 51–75 (3), 76–100 (4)

878 For each category of the score (leukocyte infiltration, inflammation extent, and crypt damage),  
 879 points were multiplied by a factor of involvement of the colonic tissue. The sum of the three  
 880 categories adds up to the total score of each section (Dieleman et al., 1998).

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**Table 2. Peak identification of the main compounds detected by UHPLC-ESI-MS.**

Peak	Retention time (min)	$\lambda_{\max}$ (nm)	$[M+H]^+$	Other ions in the spectrum	Identified compound
1	2.71	204, 218, 260	288		Cyanidin
2	3.73	218, 236, 324	355	377, 711	5-O-Caffeoylquinic acid
3	7.49	219, 238, 325	355	377, 711	Chlorogenic acid (3-O-caffeoylquinic acid)
4	9.1	227, 280	579	427, 289	Procyanidin B2
5	9.45	224, 279	291	147, 139, 123	Epicatechin
6	12.24	280	867	579	Procyanidin C1
7	13.37	219, 280	1155	287, 413, 575	Cinnamtannin A2
8	15.42	216, 269, 338	579	433, 313	Vitexin 2-O-rhamnoside
9	15.68	206, 262, 348	415	397, 367, 283	Pinnatifinoside A
10	16.13	220, 256, 353	465	303	Hyperoside
11	16.52	202, 257, 353	303	621	Isoquercetin
12	19.85	268, 337	433	621	Apigenin-C-hexoside

895  $\lambda_{\max}$  is the local maximum absorbance in the UV spectrum.  $[M+H]^+$  provided the m/z value of the  
896 precursor ion. Other ions provided the m/z value from fragments detected in the mass spectrometry.

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Table 3. Macroscopic and microscopic parameters.

Largest necrotic lesion					
Parameter	<i>C</i>	<i>CC</i>	<i>H</i>	<i>HC</i>	<i>MC</i>
Width (cm)	0a	2.04 ± 0.31b	0a	1.05 ± 0.28b	1.13b ± 0.27b
Length (cm)	0a	2.38 ± 0.17b	0a	0.96 ± 0.22c	0.92 ± 0.27c
Area (width x length, cm <sup>2</sup> )	0a	5.02 ± 1.13b	0a	1.31 ± 0.53a	0.72 ± 0.27a
Lesion/colon length (%)	0a	11.51 ± 1.40b	0a	4.67 ± 1.20a	4.83 ± 1.75a
Histology*					
Leukocyte infiltration	0a	8.20 ± 1.80b	0a	7.33 ± 0.98b	4.85 ± 0.73b
Inflammation extent	0a	7.40 ± 1.69b	0a	7.00 ± 1.12b	5.14 ± 0.59b
Crypt damage	0a	8.00 ± 2.21b	0a	6.00 ± 2.11b	4.74 ± 0.60b
TOTAL (sum)	0a	23.60 ± 5.41b	0a	16.40 ± 1.03b**	14.71 ± 1.79b

906 *C*: control, *CC*: control colitis, *H*: hawthorn, *HC*: hawthorn colitis, *MC*: mesalamine colitis.

907 Results are presented as mean ± SEM. One-way ANOVA followed by Tukey. Different letters

908 indicate statistical significance ( $p < 0.05$ ). \*Score calculation: each parameter (crypt damage,

909 inflammation extent, and leukocyte infiltration) was multiplied by the factor of involvement of the

910 mucosa, and the total is the sum of the three results (Dieleman et al., 1998). \*\*The sum of this

911 group was not  $20.33 \pm 4.21$ , as the sum of the three parameters suggests (sum of the scores of

912 leukocyte infiltration, inflammation extent, and crypt damage); this happened due to an outlier only

913 found in the statistical analysis of the TOTAL.

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**Table 4. Antioxidant capacity of the serum, inflammatory profile, and activity or****concentration of oxidative metabolism enzymes.**

<b>Antioxidant analyses (serum)</b>					
<b>Parameter</b>	<b>C</b>	<b>CC</b>	<b>H</b>	<b>HC</b>	<b>MC</b>
FRAP	0.62 ±	0.55 ±	0.60 ±	0.56 ±	0.59 ±
(µmol Trolox/mL)	0.06a	0.04a	0.05a	0.01a	0.06a
ORAC	4.31 ±	3.82 ±	7.26 ±	5.63 ±	4.04 ±
(µmol Trolox/mL)	0.71a	0.81a	2.08a	0.47a	0.65a
<b>Inflammatory profile (colon and serum)</b>					
MPO (colon)	9.40 ±	39.80 ±	9.80 ±	11.67 ±	8.66 ±
(U/g protein)	0.50a	11.27b	1.49a	2.74a	0.61a
IL-1β (colon)	111.20 ±	529.90 ±	81.34 ±	271.30 ±	208.00 ±
(ng/g protein)	3.25a	127.30b	14.20a	39.32a	39.78a
IL-1β (serum)	0.40 ±	0.44 ±	0.44 ±	0.47 ±	0.34 ±
(ng/mL)	0.04a	0.08a	0.06a	0.09a	0.03a
TNF-α (colon)	86.27 ±	77.64 ±	85.73 ±	106.50 ±	105.80 ±
(ng/g protein)	2.37a	16.17a	4.25a	15.38a	19.70a
TNF-α (serum)	0.07 ±	0.17 ±	0.06 ±	0.13 ±	0.08 ±
(ng/mL)	0.01a	0.06a	0.01a	0.06a	0.01a
<b>Oxidative metabolism enzymes (colon)</b>					
CAT	87.60 ±	211.00 ±	56.00 ±	86.50 ±	209.90 ±
(U/g protein)	10.49a	33.48b	13.33a	20.63a	21.85b
GR (consumed	106.20 ±	41.60 ±	198.00 ±	324.50 ±	58.71 ±
NADPH/min/g	19.7ab	5.11a	76.51bc	19.47c	9.38a
protein)					



SOD	144.40 ±	56.00 ±	142.80 ±	105.00 ±	54.67 ±
(SOD/g protein)	45.57 <b>a</b>	16.83 <b>a</b>	43.34 <b>a</b>	32.83 <b>a</b>	4.81 <b>a</b>
TBARS (nmol	66.78 ±	77.58 ±	37.66 ±	68.30 ±	56.41 ±
MDA/g protein)	12.42 <b>a</b>	24.24 <b>a</b>	10.84 <b>a</b>	10.49 <b>a</b>	8.02 <b>a</b>
GSH (nmol GSH/mg	14.25 ±	9.39 ±	16.70 ±	11.22 ±	13.74 ±
protein)	2.85 <b>a</b>	4.06 <b>a</b>	2.08 <b>a</b>	2.21 <b>a</b>	1.94 <b>a</b>

921 *C*: control, *CC*: control colitis, *H*: hawthorn, *HC*: hawthorn colitis, *MC*: mesalamine colitis. FRAP:  
922 ferric reducing antioxidant power, ORAC: oxygen-radical absorbing capacity. CAT: catalase, GR:  
923 glutathione reductase, MPO: myeloperoxidase, SOD: superoxide dismutase, TBARS: thiobarbituric  
924 acid reactive substances, MDA: malondialdehyde, GSH: glutathione. IL-1 $\beta$ : interleukin-1beta,  
925 TNF- $\alpha$ : tumor necrosis factor-alpha. Results are presented as mean  $\pm$  SEM. One-way ANOVA  
926 followed by Tukey. Different letters indicate statistical significance ( $p < 0,05$ ).

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**943 Fig. 1. UHPLC profile of the main identified polyphenols in the hawthorn tea**

944 Peak or relative area (%) identification: 1: Cyanidin, 2: 5-*O*-caffeoylquinic acid, 3: chlorogenic  
945 acid, 4: procyanidin B2, 5: epicatechin, 6: procyanidin C1, 7: cinnamtannin A2, 8: vitexin-2-*O*-  
946 rhamnoside, 9: pinnatifinose A, 10: hyperoside, 11: isoquercetin, 12: apigenin C-hexoside. The  
947 analysis was performed in triplicate. **A.** Peak profile of compounds from hawthorn tea by UV  
948 monitoring at 273 nm. **B.** Relative peak area (%) of compounds peaks from hawthorn tea. The  
949 relative area was calculated by dividing the peak area of each component by the sum of the peak  
950 area of the 12 identified components.

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**952 Fig. 2. Body weight, colon weight/length ratio, and appearance of necrotic lesions**

953 *C*: control, *CC*: control colitis, *H*: hawthorn, *HC*: hawthorn colitis, *MC*: mesalamine colitis.

954 Results are presented as mean  $\pm$  SEM. One-way or Two-way ANOVA followed by Tukey.

955 Different letters indicate statistical significance ( $p < 0,05$ ). **A.** Body weight alteration (%) after colitis  
956 induction (days 15 to 21). The calculation was determined in relation to day 14. **B.** Colon  
957 weight/colon length (g/cm) ratio as an inflammatory index. **C.** Photographic representation of the  
958 necrotic lesions on the colonic mucosa of rats with colitis.

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**960 Fig. 3. Photographic representation of the score of stool consistency**

961 **A.** Score 0 - Normal stools. **B.** Score 1 - Soft pellets not adhering to the anus. **C.** Score 2 - Very soft  
962 pellets adhering to the anus. **D.** Score 3 - Liquid stool; wet anus. Score based on Gommeaux et al.  
963 (Gommeaux et al., 2007) and photographic scheme based on Nascimento et al. (Nascimento, Lima,  
964 et al., 2020).

965

**966 Fig. 4. Photographic representation of the score of bleeding**

967 **A.** Score 0 – NO bleeding. **B.** Score 1 - Small spots of blood in stool; dry anal region. **C.** Score 2 -  
 968 Large spots of blood in stool; blood appears through the anal orifice. **D.** Score 3 - Deep red stool;  
 969 blood spreads largely around the anus. Score based on Gommeaux et al. (Gommeaux et al., 2007)  
 970 and photographic scheme based on Nascimento et al. (Nascimento, Lima, et al., 2020).

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### 972 **Fig. 5. Photographic representation of the distal colon histology**

973 Hematoxylin and eosin stain. Settings: 10x/0.25 magnification, 6.31x/0.25 AxioCam ICc5 (Zeiss,  
 974 Germany). Scale: 100  $\mu\text{m}$ . *C: control, CC: control colitis, H: hawthorn, HC: hawthorn colitis, MC:*  
 975 *mesalamine colitis.*

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### 977 **Fig. 6. Heatmap correlation matrix**

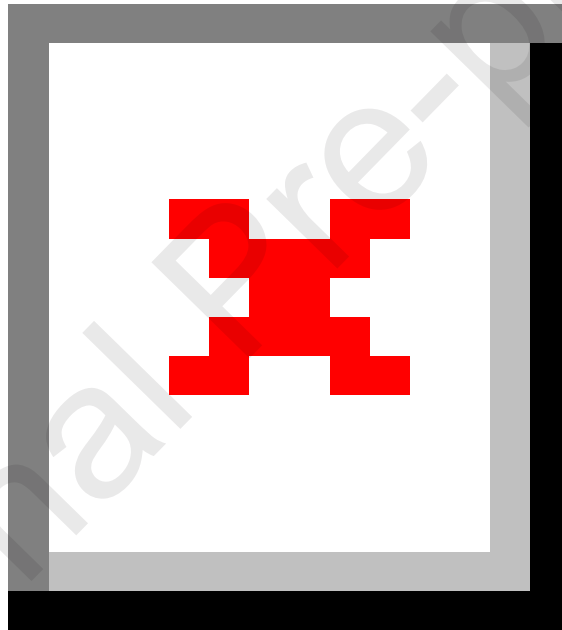
978 Pearson's correlation analysis. Correlations considered high (values between 0.7 and 1 or -0.7 and -  
 979 1) are marked with an asterisk (\*). Parameters: WEIGHT %: body weight alteration on euthanasia;  
 980 DAI DAY 1: Disease Activity Index after one day of the administration of 2,4,6-trinitrobenzene  
 981 sulfonic (peak day of symptoms); W/L COLON: colon weight/length ratio; LESION Wi: width of  
 982 the largest necrotic lesion; LESION L: length of the largest necrotic lesion; LESION A: area of the  
 983 largest necrotic lesion; LESION %: the proportion of the colon affected by the largest necrotic  
 984 lesion; HISTOLOGY T: total score of the histology; HISTOLOGY LI: score of the leukocyte  
 985 infiltration; HISTOLOGY IE: score of the inflammation extent; HISTOLOGY CD: score of the  
 986 crypt damage; CAT: catalase levels in the colon; GR: glutathione reductase levels in the colon;  
 987 MPO: myeloperoxidase levels in the colon; SOD: superoxide dismutase levels in the colon; IL-1 $\beta$ :  
 988 interleukin-1beta levels in the colon.

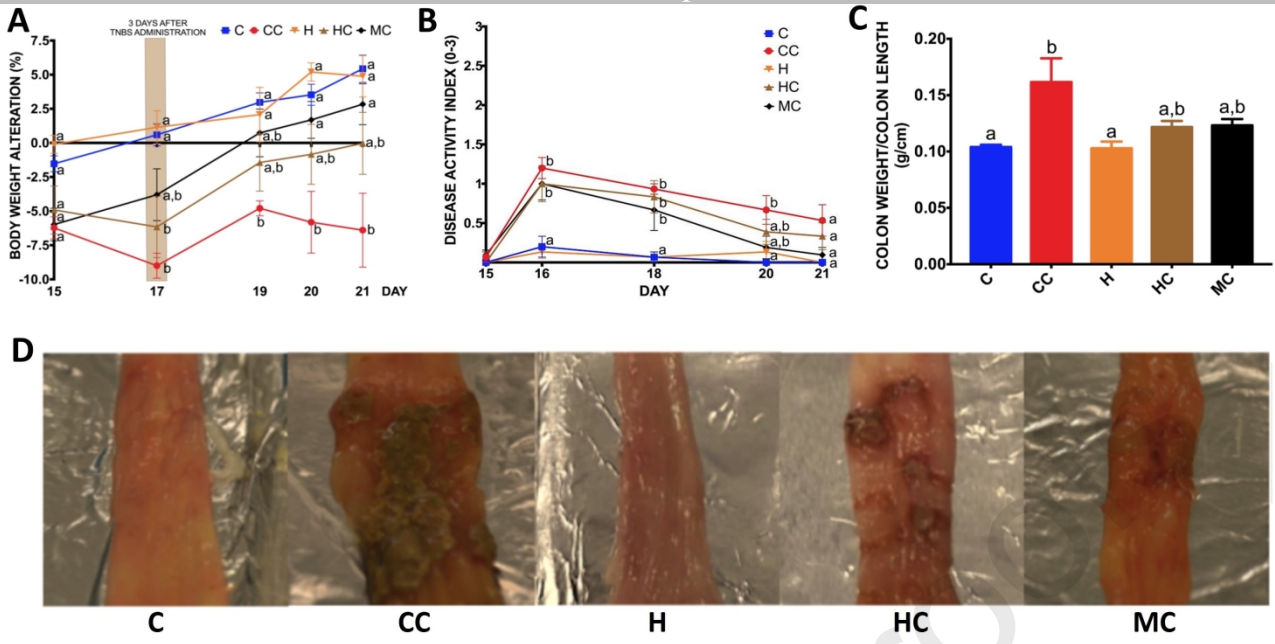
### 989 **HIGHLIGHTS**

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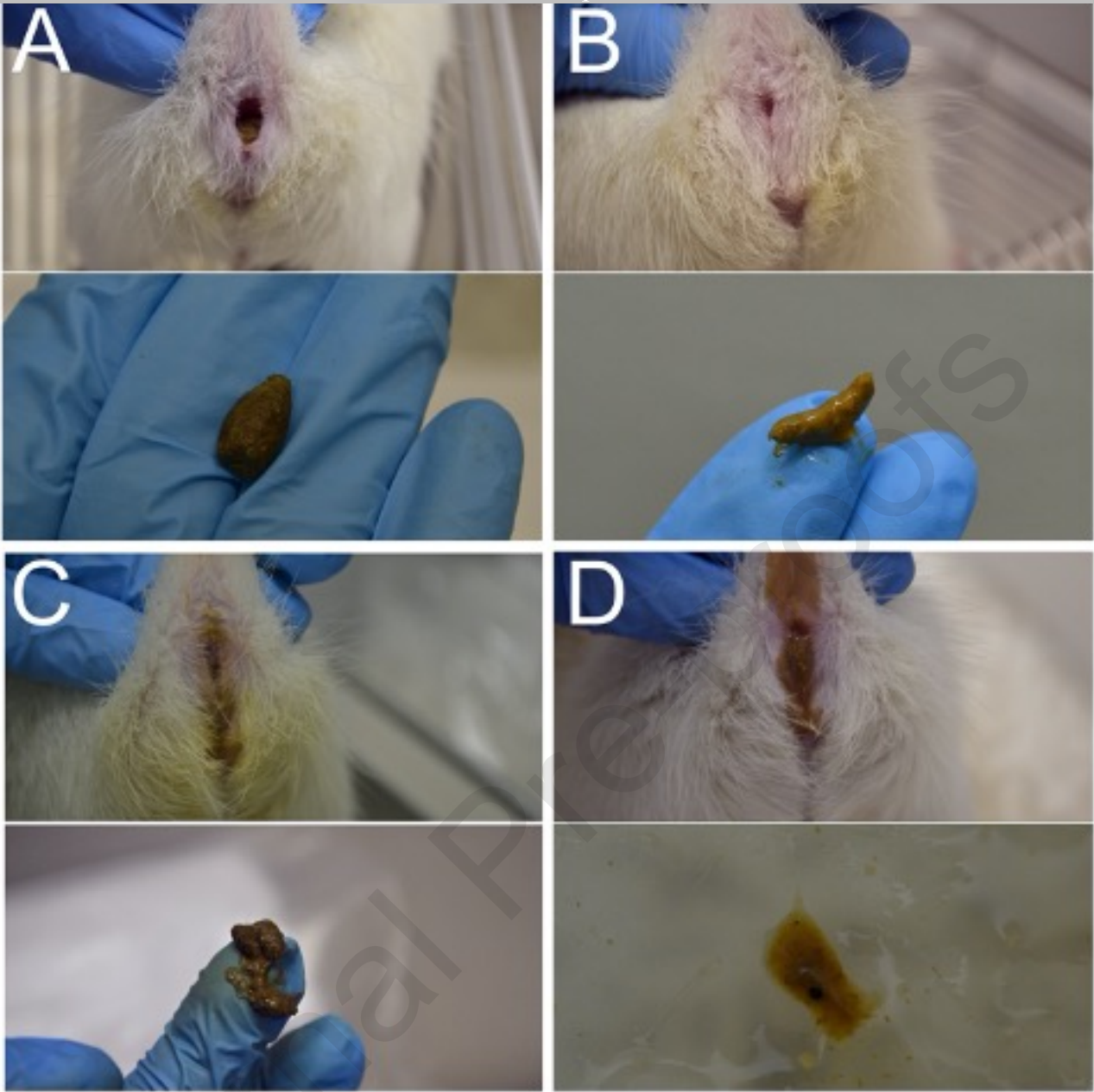
- 991 • **A tea from the leaves and flowers of hawthorn** is rich in flavonoids, especially  
 992 vitexin-2-O-rhamnoside.

- 993
- mesalamine and hawthorn tea have positive healing effects in rats with colitis.
- 994
- Hawthorn tea reduces the length and area of the brownish necrotic lesions.
- 995
- Hawthorn tea diminishes the levels of the inflammatory markers MPO and IL-1 $\beta$ .
- 996
- Hawthorn tea regulates the activity of the oxidative stress enzymes CAT and GR.
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- 998

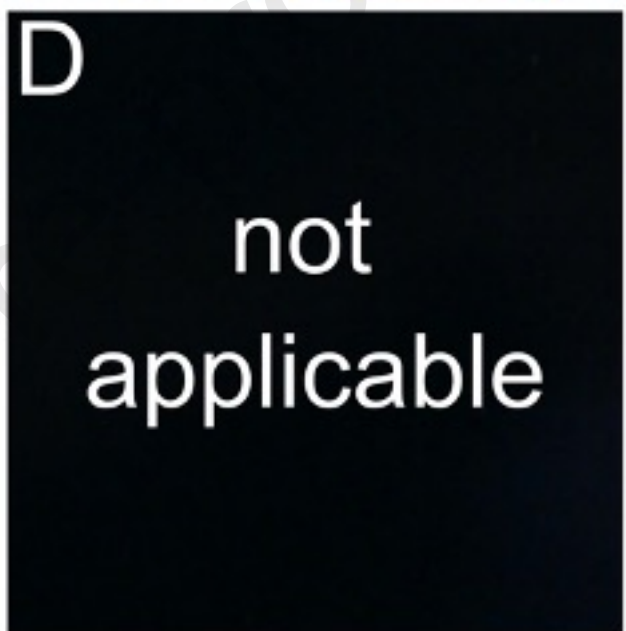




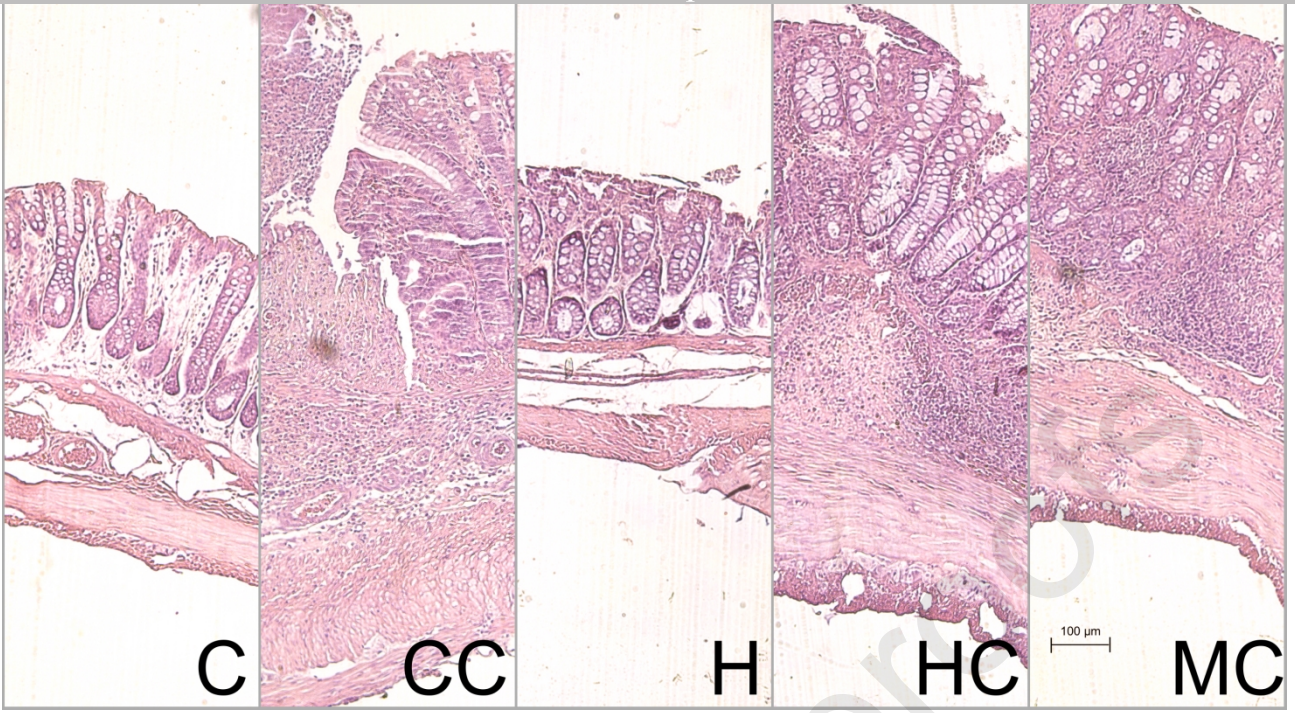
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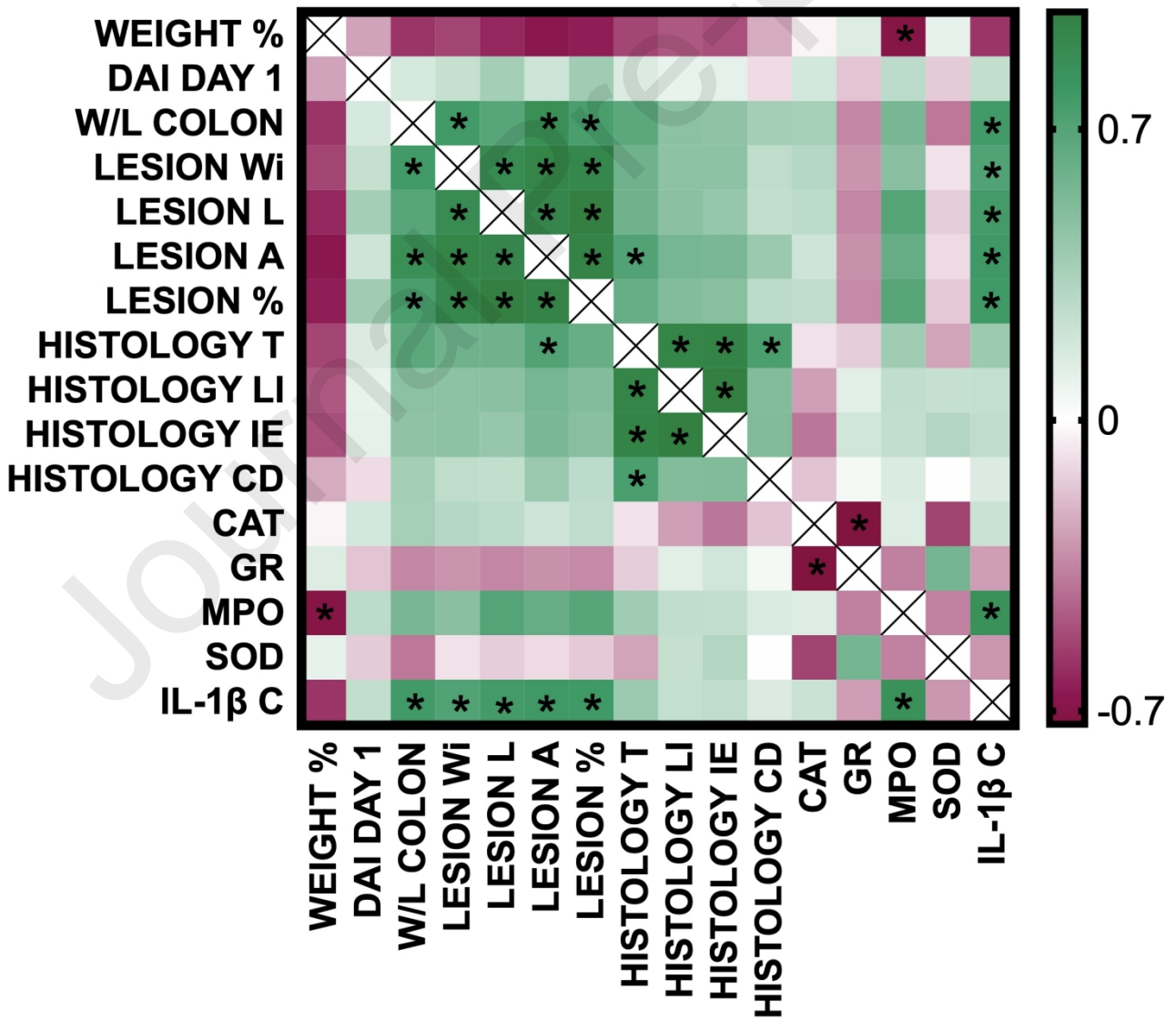
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# HAWTHORN PLANT

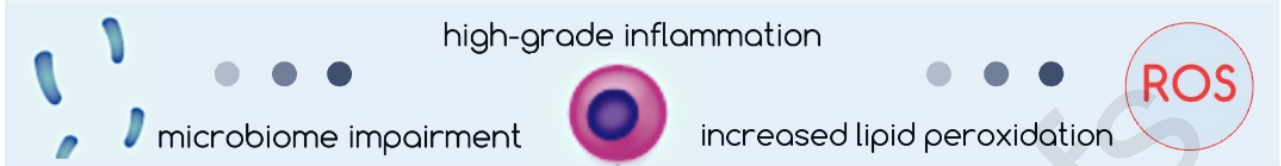


*Crataegus oxyacantha*: rich in total polyphenols, flavonoids, and proanthocyanidins

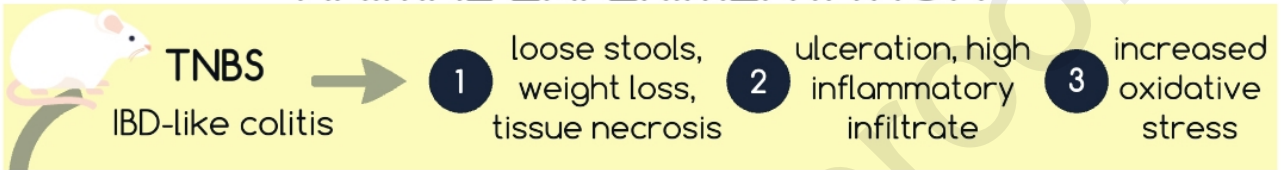


Used to treat chronic congestive heart failure. Evidence for **intestinal diseases** is still poorly known.

## INFLAMMATORY BOWEL DISEASES



## ANIMAL EXPERIMENTATION



## PREVENTIVE EFFECTS



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