Effects of *Boswellia serrata* Roxb. and *Curcuma longa* L. in an *in vitro* intestinal inflammation model using immune cells and Caco-2

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Abstract: Inflammatory bowel diseases, which consist of chronic inflammatory conditions of the colon and the small intestine, are considered a global disease of our modern society. Recently, the use of herbal therapies has increased in patients with inflammatory bowel diseases because of their effectiveness and better safety profile, compared to conventional drugs. *Boswellia serrata* Roxb. and *Curcuma longa* L. are amongst the most promising herbal drugs, however, their clinical use in inflammatory bowel diseases is limited and little is known on their mechanism of action. The aim of this work was to investigate the effects of two standardized extract of *B. serrata* and *C. longa* in an *in vitro* model of intestinal inflammation. Their impact on cytokine release and reactive oxygen species production, as well as the maintenance of the intestinal barrier function and on intestinal mucosa immune cells infiltration, has been evaluated. The extracts showed a good protective effect on the intestinal epithelium at 1 μg/ml, with *C. longa* having an anti-inflammatory mechanism of action and *B. serrata* acting as an antioxidant. In summary, these herbal products were demonstrated to be promising agents for the management of inflammatory bowel diseases by modulating *in vitro* parameters which have been identified in the clinical conditions.

Keywords: *Boswellia serrata* Roxb.; *Curcuma longa* L.; intestinal bowel diseases (IBD); Caco-2; PBMC; HMC-1.1; mast cells; cytokines; trans epithelial electrical resistance (TEER); reactive oxygen species (ROS)

1. Introduction

Inflammatory bowel diseases (IBDs) are a group of diseases very common in modern society, correlated with strong inflammatory conditions of the colon and small intestine [1]. The actual aetiology of IBDs is still unknown [2]. The incidence of IBDs in the 20th century was thought to be limited to Western countries [3], however, in the 21st century the incidence of IBDs has increased throughout the world, making it an important global disease [4].

IBDs are characterized by aberrant immune responses against microorganisms which are present in the intestine. Mast cells may play a critical role in IBDs pathogenesis, since they are typically located just beneath the intestinal mucosal barrier and can be activated by bacterial

48   antigens. These cells can potentially contribute to IBDs through their effects on immunoregulation
49   [5].
50   The current pharmacological treatments for IBDs focus on the use of drugs, such as
51   5-aminosalicylates, corticosteroids, immunosuppressive and biological agents, able to reduce
52   inflammation and related symptoms [6]. However, these drugs present some side effects such as
53   rash, nausea and vomiting, which limit their therapeutic application [7].
54   Herbal therapies have been used since ancient time to treat a wide variety of diseases [8,9] and
55   can also represent a valid alternative to conventional treatments in IBDs due to their validated
56   effectiveness and better profile of safety.
57   Among the multiple phytotherapic agents available, *Curcuma longa* L. rhizome (turmeric) and
58   *Boswellia serrata* Roxb. gum resin (boswellia) are considered among the most promising herbal drugs
59   for the management of IBDs [10].
60   According to the European Medicines Agency, *C. longa* root has a therapeutic indication
61   derived from traditional use for the relief of gastrointestinal disorders [11]. Curcuminoids are able to
62   inhibit lipoxygenases, cyclooxygenases and phospholipases and act on AP-1, STAT and NF-κB
63   pathways [12,13]. Furthermore, an important free radical scavenging activity has been associated
64   with the biological activity of turmeric [14].
65   In 2014, McCann and colleagues showed that curcumin-enriched turmeric extracts were able to
66   increase the activity of an IL-10 promoter variant associated with IBD in human embryonic kidney
67   cells [15]. Moreover, curcumin inhibited the proliferation of splenocytes as well as IL-4 and IL-5
68   secretion by CD4(+) lymphocytes in a mouse model of chemically-induced colitis [16]. The same
69   group, then, demonstrated that curcumin can attenuate the release of MIP-1α, MIP-2 and IL-1β from
70   colonic epithelial cells and macrophages, as well as the release of IL-8 from neutrophils [17].
71   According to the World Health Organization, boswellia use for the management of IBDs is
72   supported by clinical use [18]. The anti-inflammatory activity of boswellia is related to the inhibition
73   of lipoxygenases and NF-κB [19]. Furthermore, the inhibition of lipid peroxidation and the increase
74   of superoxide dismutase levels, which contribute to the antioxidant activity of boswellia, were
75   correlated to the intestinal anti-inflammatory effect observed in an *in vivo* colitis model in rats
76   [20,21].
77   Despite their biological potential, the clinical use of turmeric and boswellia in IBDs is still
78   limited and controversial because of the lack of registered drugs in many countries and the
79   variability of preparations used in clinical trials.
80   These reasons led us to investigate the *in vitro* effectiveness and the protective mechanisms of
81   action of two dry extracts of *Curcuma longa* L. rhizome (CUR), standardized in curcumin, and
82   *Boswellia serrata* Roxb. gum resin (BOS), standardized in boswellic acids, in maintaining the intestinal
83   barrier integrity, using an innovative protocol that considered their action in human epithelial
84   colorectal cells as well as immune cells (i.e. peripheral blood mononuclear cells and mast cells).

2. Results

2.1. Chemical analyses of dry extracts

87   Curcumin and boswellic acids content of dry extracts accomplished the values declared in
88   products labels.
89   Curcumin content in CUR, according to Ph. Eur. 9th method, resulted 56.85% ± 2.79%, whereas
90   total triterpenes content in BOS was 68.41% ± 3.33%.

2.2. Inflammatory model on Caco-2, PBMC and HMC-1.1: cytokines dosages

92   Firstly, we verified that, as reported in literature, intestinal inflammation is not related to
93   cytokine release by intestinal epithelial cells [22]. We used Caco-2 cells as a model of the intestinal
94   lumenal epithelum.
95   Measurement of cytokines released by Caco-2, after stimulation with a pro-inflammatory
96   concentration of bacterial lipopolysaccharide (LPS) revealed IL-6, IL-8 and IL-10 levels were not

97 modified by LPS stimulation (500 ng/ml) but TNF-α was upregulated 1.38 fold compared to
98 untreated controls (figure 1).
99

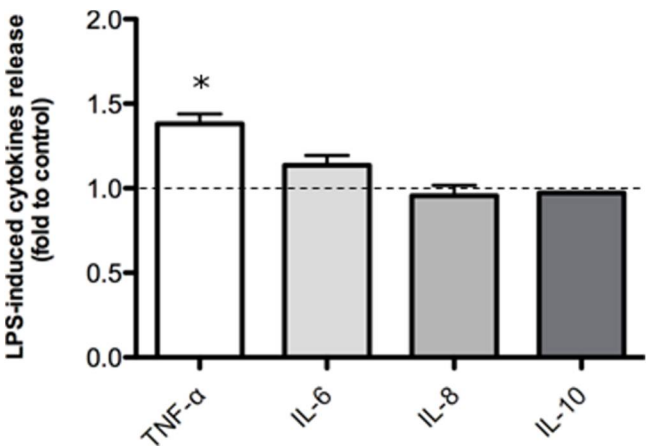
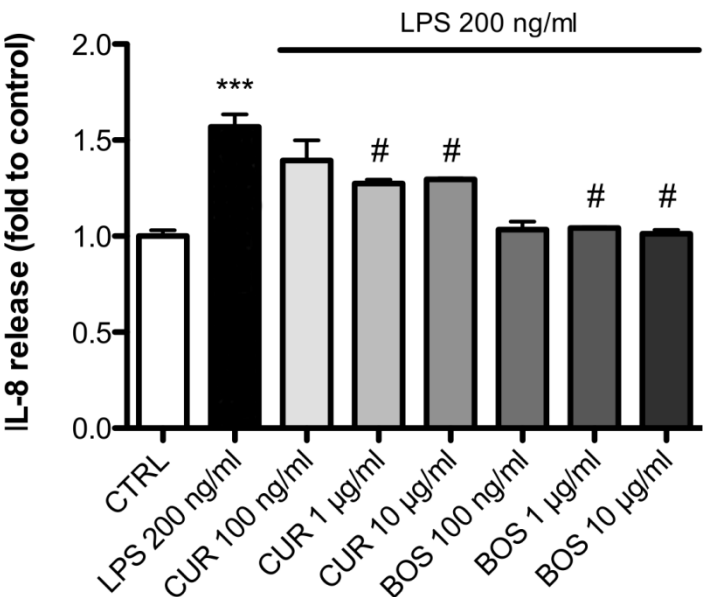
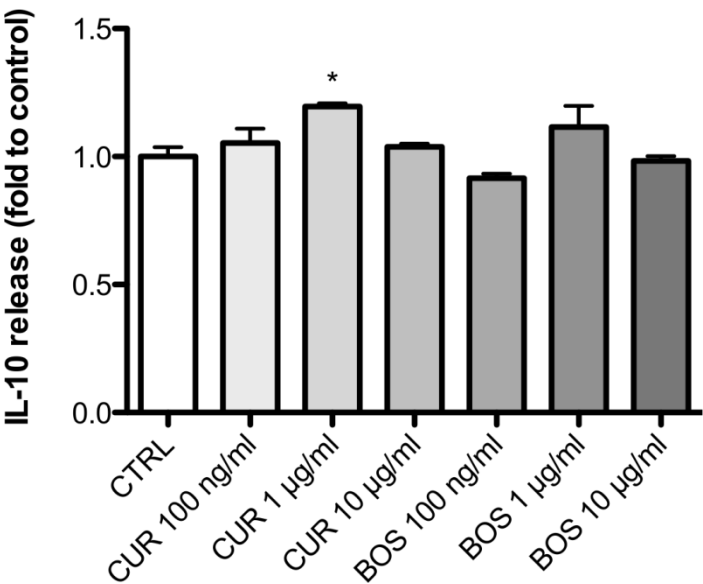



Figure 1. Cytokine release in Caco-2 cells; * p<0.05 *vs* control (set at 1).

100
101 Despite the poor release of cytokines by epithelial cells *in vitro*, cytokine levels are considered
102 pivotal in maintaining and promoting IBDs such as ulcerative colitis and Crohn's disease. The high
103 levels of cytokine production observed during intestinal inflammation are probably due to
104 infiltrating immune cells [23].
105 For this reason we focused our attention on a LPS-induced inflammation model using
106 peripheral blood mononuclear cells (PBMC) and we investigated the anti-inflammatory activity of
107 CUR and BOS.
108 The inflammatory model using PBMC confirmed a signficant impact on production of
109 cytokines following LPS stimulation. Notably TNF-α was over than 50 fold up-regulated compared
110 to the control in each experiment we performed. IL-6 also was strongly up-regulated (over 15 folds
111 compared to the control) (figure S2)
112 IL-8 up-regulation induced by LPS less striking compared to other cytokines but was
113 statistically significant and confirmed that this chemo-attractant was induced by LPS (figure .
114 Neither CUR nor BOS at any of the tested concentrations modulated LPS-stimulated TNF-α and
115 IL-6 production by PBMC (figure S1).
116 In contrast, IL-8 up-regulation induced by LPS was inhibited by both CUR and BOS (figure 2);
117 with signficant reductions seen with CUR at 1 and 10 µg/ml which inhibited IL-8 release by 14.84%
118 and 13.23%, respectively, compared to LPS-stimulated cells. At the lowest concentration used (100
119 ng/ml), CUR was not effective in inhibiting IL-8 release in stimulated PBMC.
120 Likewise, BOS inhibited IL-8 release in stimulated PBMC at each of the tested concentrations,
121 even if statistically significance was obtained only at 1 µg/ml and at 10 µg/ml, with a reduction of at
122 9.32% and 11.90%, respectively, compared to LPS groups (figure 2).

123   **Figure 2.** IL-8 release in PBMC; *** p<0.001 *vs* control; # p<0.05 *vs* stimulus.
124

125   Additionally the release of IL-10, the most representative regulatory cytokine, was monitored in
126   non-inflammatory conditions. IL-10 release by PMBC incubated with CUR was up-regulated by
127   19.52% compared to the control group. On the contrary, BOS had no effects on IL-10 (figure 3).
128

129   **Figure 3.** IL-10 release in PBMC; * p<0.05 *vs* control.
130

131   As a result of the preceding experiments with PBMC, the concentration of 1 μg/ml, which was
132   the minimum effective concentration, was chosen for further experiments.
133   TNF-α release modulation after LPS stimulation and CUR and BOS treatment on Caco-2 cells
134   was re-evaluated (figure 4). It was noticeable that incubation with either CUR or BOS was effective
135   in inhibiting LPS-induced TNF-α in Caco-2 by 25.60% and 13.82%, respectively.

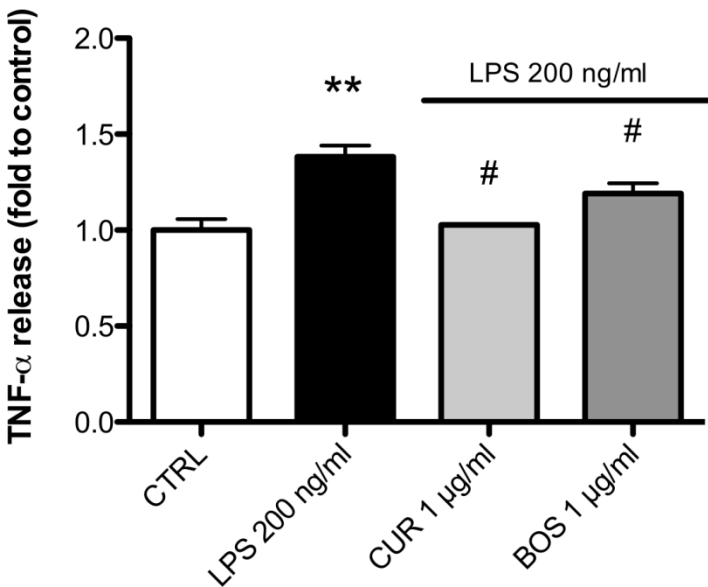



Figure 4. TNF-α release in Caco-2; ** p<0.01 *vs* control; # p<0.05 *vs* stimulus.

Afterwards, in order to better understand the modulation of cytokines observed in PBMC, which are composed of several immune cell subpopulations, we analysed the behaviour of a specific immune cell line involved in IBDs. Hence, we evaluated the release of inflammatory cytokines such as IL-6, IL-8, IL-10 and TNF-α by mast cells (HMC-1.1), as these are believed to play a crucial role in inflammatory conditions.

Stimulation of HMC-1.1 cells with phorbol 12-myristate,13-acetate (PMA) resulted in increased secretion of TNF-α, IL-6, and IL-8 by 4, 8 and 4,5 folds, respectively, compared to the untreated control.

Interestingly, IL-6, the most abundant PMA-induced cytokine produced by HMC 1.1 was significantly inhibited by both CUR and BOS, by 39.44% and 39.06%, respectively (figure 5).

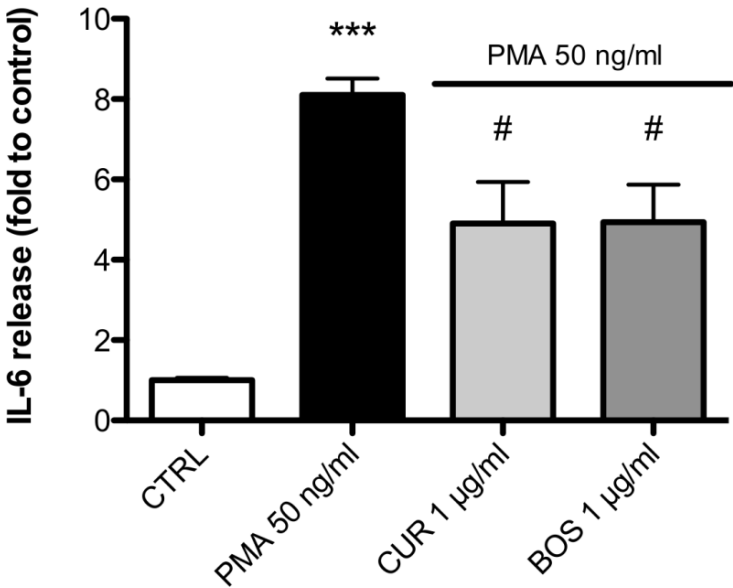



Figure 5. IL-6 release in HMC-1.1; *** p<0.001 *vs* control; # p<0.05 *vs* stimulus.

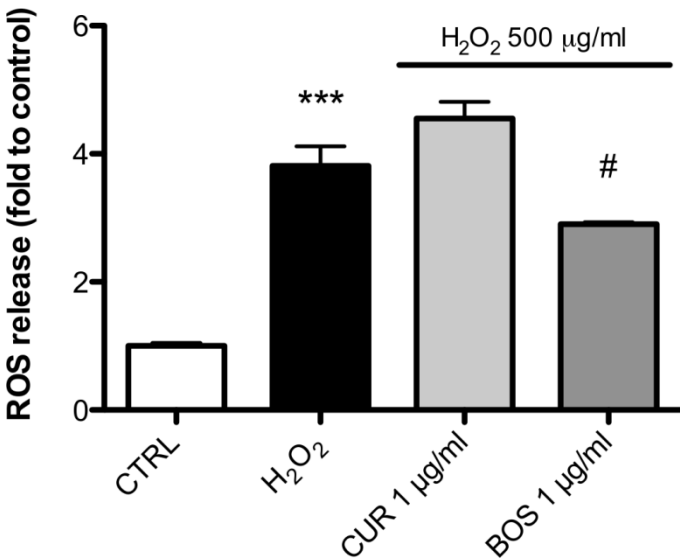
Neither CUR nor BOS at each of the tested concentrations modulated the release of TNF-α and IL-8 in HMC-1.1 stimulated by PMA (not shown).

IL-10 release was monitored too, but no variation was detected after the treatment of HMC-1.1 cells with CUR and BOS (not shown).

156   *2.3. Measurement of ROS production*

157   Since reactive oxygen species (ROS) production is common in every cell line and it is a typical
158   phenomenon involved in inflammatory conditions [24], we analysed the modulation of ROS by CUR
159   and BOS in Caco-2 cells.

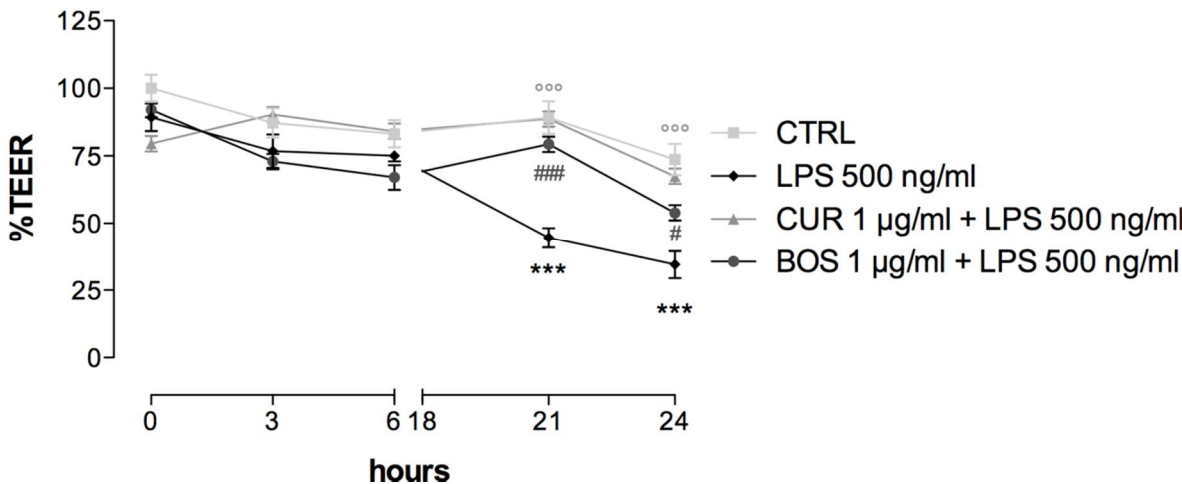
160   Our analysis showed that H_2O_2 increased the ROS production in Caco-2 by 3.83 fold compared
161   to the control. BOS 1 μg/ml decreased the ROS production by 24.33% compared to H_2O_2, while CUR 1
162   μg/ml was ineffective in inhibiting ROS production after H_2O_2 stimulation (figure 6).

163

164   **Figure 6.** ROS release in Caco-2 cells; *** $p<0.001$ *vs* control; # $p<0.05$ *vs* stimulus.

165

166   *2.4. Intestinal permeability: TEER measurements*

167   As described before, high levels of cytokines and leukocyte activation could be better framed as
168   a consequence of an alteration in intestinal mucosa integrity and, thus, of increased epithelial
169   permeability. Hence, we evaluated the integrity of the intestinal epithelium barrier by measuring the
170   trans-epithelial electrical resistance (TEER) in LPS-stimulated Caco-2 cells, in order to assess the
171   protective effect of BOS and CUR (figure 7).

172

173   **Figure 7.** TEER measurement in Caco-2 cells. *** $p<0.001$ stimulus *vs* control; °°° $p<0.001$ CUR *vs*
174   stimulus; ### $p<0.001$ BOS *vs* stimulus; # $p<0.05$ CUR *vs* stimulus.

175

176   TEER is expressed as the percentage of resistance, normalized to the initial value.

177   The treatment with LPS decreased the TEER value by 55.04% and 65.51% compared to the not
178   treated control, after 21 and 24 hours, respectively.

179   CUR almost completely protected the intestinal barrier, increasing the TEER value by 43.73%
180   after 21 hours and by 32.98% after 24 hours, compared to LPS.

181   The pretreatment with BOS enhanced the TEER value by 34.33% and 19.41%, after 21 and 24
182   hours, respectively, compared to LPS, thus, significantly reverting the epithelial damage caused by
183   the inflammatory stimulus.

184   *2.5. Leukocytes infiltration: PBMC adhesion assay*

185   Immune cell infiltration is a critical event for the establishment of intestinal inflammation. By
186   developing a co-culture between LPS-stimulated Caco-2 and PBMC, we tried to simulate intestinal
187   inflammation, evaluating the effect of CUR and BOS pretreatment.

188   LPS stimulation resulted in a readily detectable leukocyte infiltration, represented by the blue
189   stained spots (figure 8b), that was inhibited by the treatment with CUR or BOS (figure 8c and 8d).

190   In agreement with the TEER measurements, the adhesion assay demonstrated a strong
191   protective effect of CUR and BOS on intestinal epithelium.

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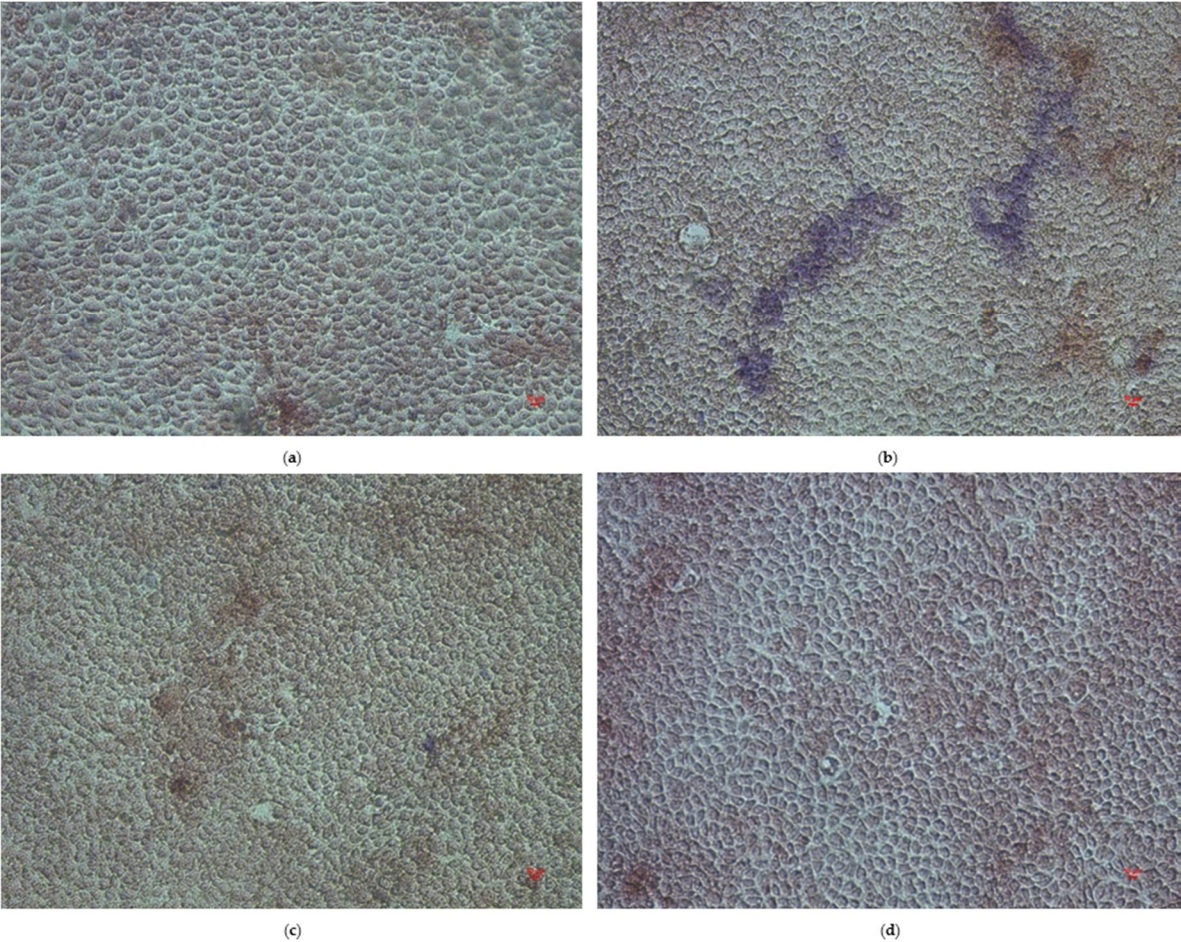
Figure 8. PBMC adhesion assay: (**a**) control, (**b**) LPS 500 ng/ml, (**c**) CUR 1 µg/ml + LPS 500 ng/ml, (**d**) BOS 1 µg/ml + LPS 500 ng/ml.

193

3. Discussion

195   IBDs are strictly correlated with intestinal barrier dysfunctions, which lead to a strong antigenic
196   response, characterized by oxidative damage and inflammation [25].

197   In this study CUR and BOS both demonstrated a strong protective effect on the epithelial
198   barrier by increasing TEER values and reducing immune cells infiltration. These data are in

199 agreement with our previous experiments for boswellia [26] but also suggest a novel interesting
200 biological effect of turmeric.
201   The co-culture of Caco-2 cells and PBMC resulted in a dynamic and more realistic picture of
202 bowel inflammatory conditions, by showing how blood mononuclear cells participate in the
203 infiltration process linked to modified integrity of inflamed intestinal epithelial cells.
204   In our model, apart from a slight increase in TNF-α levels, we could not detect a change in
205 cytokine levels in LPS-stimulated Caco-2 compared with the non-treated control. Indeed, intestinal
206 inflammation appears not to be related to cytokine release by epithelial cells. In Caco-2, this is
207 possibly due to the low level of TLR-4 receptors expressed. These receptors, in fact, are mainly
208 expressed at the crypts rather than at the lumen, and mediate the response to the LPS stimulus,
209 resulting in NF-κB signal activation [22]. On the contrary, immune cells are largely responsible for
210 intestinal cytokines accumulation in IBDs [27]. For these reasons, we used immune cells in order to
211 simulate an inflammatory environment, with the aim of better understanding the anti-inflammatory
212 mechanism(s) of action of CUR and BOS in IBDs.
213   The comprehensive analysis of cytokines modulated by CUR and BOS after inflammatory
214 stimuli on the different cell types revealed that the tested samples had some peculiar
215 anti-inflammatory effectiveness at the concentration of 1 μg/ml. In particular, both CUR and BOS
216 were effective in reducing IL-6 release from mast cells, CUR was more effective in reducing TNF-α
217 release in Caco-2, whereas BOS was more effective in reducing IL-8 release in PBMC. The reduction
218 in IL-8 release from PBMC confirmed the results obtained by Larmonier and colleagues, who
219 observed a similar activity in neutrophils, even if in our model we obtained different results on IL-1β
220 [17]. Moreover, the increase in IL-10 in CUR-treated PBMC is consistent with the results obtained by
221 McCann and collaborators [15].
222   In the light of these data, we assume that the modulation of the biosynthesis and release of
223 cytokines, although important and significant, cannot actually be considered as the main mechanism
224 of action of these phytotherapics in IBDs.
225   The antioxidant properties of BOS, rather than its anti-inflammatory effects, may better explain
226 the protective effect of this extract in intestinal inflammation and could be, partly at least, marked as
227 a specific mechanism of action, as previously reported [20,21,26]. CUR was not able to protect Caco-2
228 cells from H_2O_2-induced ROS accumulation, even if turmeric is thought to possess an important
229 radical scavenging activity [14].
230   The inhibition of leukocytes infiltration, along with permeability maintenance and cytokine and
231 ROS level reduction, observed with CUR and BOS treatment, revealed the beneficial effect of these
232 phytotherapics on intestinal inflammation, which occurs through multiple mechanisms of action.
233 Moreover, comparing our findings with previously reported data [26] and preliminary
234 investigations conducted in our laboratories (data not shown), we obseved no statistically significant
235 difference between the activity of BOS and CUR compared to of acetyl-11-keto-β-boswellic acid
236 (AKBA) and curcumin (the main constituents of boswellia and turmeric, respectively). This confirms
237 that using the whole herbal phytocomplex, which may have an high content of AKBA and curcumin,
238 can lead to similar therapeutic effects, but may that this may also be achieved by administrating a
239 lower concentration of active principles.

240 **4. Materials and Methods**

241 *4.1. Extracts preparation and chemical analysis*

242   A commercial dry extract of *Curcuma longa* L. rhizome, standardized to contain 50% curcumin
243 was bought in a pharmacy in Siena. Curcumin and curcuminoids content were quantified according
244 to the spectrophotometric method described in the European Pharmacopoeia (Ph. Eur. 9th) [7].
245   The dry extract of *Boswellia serrata* Roxb. gum resin, standardized to contain 65% boswellic acids
246 was gently provided by Prof Monica Montopoli, University of Padua.
247   Experiments were conducted in triplicate. All the chemicals and solvents were purchased from
248 Sigma-Aldrich, Milan.

249   4.1.1. Curcumin quantification

250   60 ml of glacial acetic acid were added to 100 mg of CUR and the solution was heated at 90 °C
251   for 60 minutes under reflux. 2 g of boric acid and 2 g of oxalic acid were added and the solution was
252   heated at 90 °C for 10 minutes under reflux.
253   The solution was cooled to room temperature and volume adjusted to 100 ml with glacial acetic
254   acid. 1 ml of the solution was diluted to 50 ml with glacial acetic acid and absorbance recorded at 530
255   nm using a spectrophotometer SAFAS UV MC2. Plastic cuvettes (1 cm optical path length) were
256   used.
257   Curcumin content was calculated according to Ph. Eur. 9th as follows:

258   *Curcumin % = (absorbance x 0,426)/weight (g) x dilution factor*

259   4.1.2. Total triterpenes quantification

260   Boswellic acids are correctly quantified by HPLC, but in this work, considering the recent
261   analysis performed at University of Padua [26], we confirmed the declared content of boswellic acids
262   using the rapid colorimetric assay published by Fan and He (2006) for total triterpenes [28].
263   BOS was used as a 10 mg/ml ethanolic solution (96% V/V). 10 µl of the sample solution were
264   added to 190 µl glacial acetic acid. 300 µl of a 5% m/V vanillin in glacial acetic acid solution were
265   added and mixed for 30 seconds, before adding 1 ml of concentrated perchloric acid.
266   The mixture was heated at 70 °C for 40 minutes and, after cooling, the volume was adjusted to 5
267   ml.
268   Absorbance was recorded at 548 nm and quantification of total triterpenes in the extract
269   calculated according to the calibration curve obtained using analytical grade ursolic acid.

270   *4.2. Cell cultures*

271   4.2.1. Peripheral blood mononuclear cells

272   Peripheral blood mononuclear cells (PBMC) were isolated from blood obtained from 3 different
273   healthy volunteers by density gradient centrifugation, using Histopaque®-1077 (Sigma-Aldrich,
274   Italy) [29]. Briefly, Histopaque and freshly collected heparinized blood were added in a 15 ml tube
275   (ratio 3:5) and spinned at 325 G for 15 minutes. Lymphomonocytes ring was recovered in a 15 ml
276   tube and diluted to volume with saline solution. Up to three wash were performed by spinning at
277   240 G for 10 minutes, removing supernatant, resuspending cell pellet and diluting to volume with
278   saline solution. PBMC were suspended in a culture medium consisting of RPMI 1460 medium with
279   1% L-glutammine and 1% penicillin/streptomycin solution. Cell counting was performed using a
280   hemocytometer by Trypan blue staining and the PBMC were used immediately for experiments.
281   Incubation times were conducted at 37 °C, with 5% CO_2.

282   4.2.2. Mast cells

283   HMC-1.1 were used for experiments on mast cells [30]. An aliquot of HMC-1.1 cells had previously
284   been generously gifted by Dr Butterfield to PTKS allowing us to conduct *in vitro* mast cell
285   investigations. Cells were cultured in IMDM medium (Gibco, UK) supplemented with 10% calf
286   serum (Hyclone, Logan, Utah), using 162 cm^3 cell culture flasks (Corning Incorporated, Costar®,
287   USA) and incubated at 37 °C with 5% of CO_2. Cells were splitted and collected in 6 wells culture
288   plates (Corning Incorporated, Costar®, USA) at a density of 1×10^6 cells/ml. Each well contained 3
289   ml of cell suspension. The HMC-1.1 cells were maintained in IMDM medium, supplemented with
290   10% calf serum, plus alpha thioglycerol (Sigma, UK).. Cell counting was performed using Countess
291   II Life Technologies Cell Counter (Countess™ Invitrogen, USA), by Trypan blue staining.

292   4.2.3. Intestinal epithelium cells

293   Caco-2 cells were used as a stable *in vitro* model for the intestinal epithelium [31]. Cells were
294 obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM
295 supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin
296 antibiotic (Sigma-Aldrich, Italy). Cells were maintained under a humidified atmosphere of 5% CO_2
297 in air, at 37 °C [25,32].

298 *4.3. Evaluation of the anti-inflammatory activity*

299   An *in vitro* inflammation model was set up for each cell line.
300   Samples were solubilized in ethanol 85% V/V (Sigma-Aldrich, Italy) and then diluted in cell
301 culture medium at the concentration of 100 ng/ml, 1 μg/ml and 10 μg/ml, according to preliminary
302 conduced cell viability tests (data not shown).
303   PBMC ($1x10^6$ cells/ml) were seeded in 24 well plates and stimulated with LPS from Gram –
304 (from *Salmonella enteridis*, Sigma-Aldrich, Italy) at a concentration of 200 ng/ml.
305   HMC-1.1 ($1x10^6$ cells/ml) were seeded in 24 well plates and stimulated with PMA
306 (Sigma-Aldrich, Italy) at a concentration of 50 ng/ml.
307   Caco-2 ($4x10^4$) were seeded in 24 well plates, cultured to reach the confluence, and stimulated
308 with LPS (500 ng/ml).
309   A pre-treatment of 1 hour with BOS or CUR was administered before the stimulation.
310   After the incubation time, samples were frozen (at -80 °C) and thawed for 3 times and the
311 supernatants were collected for analysis.
312   Non-competitive sandwich ELISA kit (Biolegend e-Bioscience DX Diagnostic, Italy) were used
313 for the dosages of TNF-α, IL-6, IL-8, IL-10, following the procedure reported in the datasheet.
314 Absorbance was recorded at 450 nm using a SAFAS MP96 spectrophotometer.

315 *4.4. Measurement of ROS production*

316   ROS were quantified using 2',7'-dichlorofluorescin-diacetate (H_2-DCF-DA, Sigma-Aldrich, St
317 Louis, MO, USA), as previously described [17]. Upon cleavage of the acetate groups by intracellular
318 esterase and oxidation, the H_2−DCF-DA is converted to the fluorescent 2',7'-dichlorofluorescein
319 (DCF). Briefly, the intestinal cells were seeded into 96-well plates and allowed to adhere overnight.
320 ROS level was measured after the exposure to BOS (1 μg/ml) or to CUR (1 μg/ml) for 24 h.
321 Treatments were removed and H_2DCF-DA was added to obtain a final concentration of 50 μM in
322 each well. The plate was incubated for 30 minutes at 37°C and washed with phosphate-buffered
323 saline (PBS). DCF fluorescence intensity was measured at excitation 485 nm - emission 535 nm, using
324 VICTOR™X3 Multilabel Plate Reader (PerkinElmer), before and after the addition of 500 μM H_2O_2
325 on each well.

326 *4.5. Measurement of trans-epithelial electric resistance (TEER)*

327   The efficiency of the barrier functions was evaluated by measuring TEER using a voltmeter [26].
328   Caco-2 cells were placed in Transwells (transparent PET membrane: 0.4 μm pore size; BD
329 Falcon) as previously described [26].
330   The integrity of the cells were monitored by measuring the trans-epithelial electric resistance of
331 the mono-layer at confluence from day 14° to day 20° since cells were seeded. When a stable value
332 was reached, cells were treated with BOS (1 μg/ml) and CUR (1 μg/ml) in the appropriate wells.
333   After 24 hours of pre-treatment, an inflammatory stimulus (LPS 500 ng/ml) was given.
334   HBSS buffer was used in order to obtain from the instruments stable values of the different
335 treatments [33,34].
336   TEER measurements were carried out at 3, 6, 21 and 24 hours, after the stimulation.

337 *4.6. Adhesion assay*

338   A simulation of inflammation-induced leukocytes infiltration on intestinal mucosa was
339 performed developing a cell-cell adhesion assay of PBMC to Caco-2 cells, slightly modifying the

340 method published by Seo and colleagues [35]. Hematoxylin-eosin combination was chosen as a
341 simple and efficient staining method [36].

342   Briefly, glass cover slips (VWR, Italy) were preliminary washed in ethanol (Sigma Aldrich,
343 Italy), sterilised using a flame, covered with gelatine (Sigma-Aldrich, Italy) and incubated for 30
344 minutes.

345   Afterwards the gelatine in excess was removed and Caco-2 cells were seeded at the density of
346 $1.5x10^5$ cells/well.

347   Once they reached confluence, cells were pre-treated with BOS (1 μg/ml) and CUR (1 μg/ml) for
348 1 hour and then exposed to LPS 500 ng/ml for 24 hour.

349   PBMC ($3x10^5$) were co-cultured with Caco-2 for 1 hour and then washed with PBS.

350   Images were obtained using eclipse Ti-s (Nikon) microscope.

351 *4.7. Statistical Analysis*

352   All experiments were performed in triplicate in two independent repetitions (n=6).

353   The statistic differences between groups were determined by the analysis of the variance (one
354 way ANOVA). Values are expressed in the range of +/- standard deviation and $p < 0.05$ was
355 considered statistically significant. Graphs and calculations were performed using GraphPrism®.

356 **5. Conclusions**

357   Currently, a standardized protocol with universal efficacy for the treatment of chronic intestinal
358 diseases doesn't exist.

359   Corticosteroids are commonly prescribed during the acute phases of the disease.
360 5-aminosalicylates, immunomodulatory therapy or anti-TNF α antibodies can be used in the
361 remission phases, as an alternative or in association with corticosteroids.

362   All these drugs share considerable side effects and a quite low therapeutic compliance.
363 Therefore, the aim of this study was to evaluate new active principles with the perspective of
364 identifying new and effective treatments for IBDs with a good safety profile.

365   *In vitro* studies and animal models largely contributed to the current comprehension of the
366 inflammatory mechanisms at intestinal level. Those mechanisms include a loss of membrane
367 integrity, ROS and cytokines accumulation and the hyper-activation of immune system, particularly
368 referring to mast cells.

369   In the attempt to evaluate new agents for IBDs treatment, the development of inflammatory
370 models which consider all of these elements is mandatory.

371   In this work, an original *in vitro* approach, using various human cell lines and innovative but
372 validated methods, was proposed.

373   Turmeric and boswellia, which are principally characterized by the anti-inflammatory activity
374 and by a wide and not specific anti-oxidant effect, demonstrated to be promising agents for the
375 management of IBDs, by modulating not only the parameters indicative of dysfunction in the *in vitro*
376 models used (i.e. cytokines release and ROS production), but also the ones identified in the clinical
377 manifestation of IBDs (i.e. loss of intestinal epithelium integrity and immune cells infiltration), at
378 concentration which can be plausibly reached at intestinal level.

379   Turmeric extract confirmed to possess anti-inflammatory capacity even at low concentrations
380 and revealed itself as an extraordinary protective agent towards the intestinal epithelium integrity.
381 Boswellia extract showed comparable efficacy in the proposed model, exhibiting a lower
382 anti-inflammatory effect which is counterbalanced by the strong anti-oxidant activity. Moreover,
383 given the peculiar mechanism of action of each extract, it is likely that the association between
384 turmeric and boswellia may act in a synergistic fashion, improving the therapeutic effectiveness.
385 Thus, it is hopefully that future studies will be conducted using a mixture of extracts.

386   This study must be considered only as a starting point in the developing process of these herbal
387 drugs for IBDs. In that respect, it would be necessary to carry out further molecular and
388 pharmacological investigations and to provide extracts and formulation capable of guaranteeing a
389 good bioavailability, which is one of the main complications of these phytotherapics.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: TNF-α (**a**), IL-6 (**b**) and IL-8 (**c**) release induced by different concentrations of CUR and BOS in PBMC. No inflammatory effect was observed; Figure S2: TNF-α (**a**) and IL-6 (**b**) release induced by different concentrations of CUR and BOS in LPS-stimulated PBMC. No anti-inflammatory effect was observed. ** p<0.01 *vs* control; *** p<0.001 *vs* control; Figure S3: IL-6 (**a**) and IL-10 (**b**) release induced by CUR and BOS in HMC-1.1 cells. No effect was observed; Figure S4: TNF-α (**a**) and IL-8 (**b**) release induced by CUR and BOS in PMA-stimulated HMC-1.1 cells. No anti-inflammatory effect was observed. ** p<0.01 *vs* control; *** p<0.001 *vs* control; Figure S5: Effect of CUR and BOS on ROS production in Caco-2 cells; Figure S6: Effect of CUR and BOS on TEER measurements in Caco-2 cells.

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