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An Hydroalcoholic Chamomile Extract Modulates Inflammatory and Immune Response in HT29 Cells and Isolated Rat Colon

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Inflammatory bowel diseases (IBDs) are chronic disorders characterized by disruption and ulceration of the colonic mucosa or of any part of the digestive tract (Crohn's disease). Antioxidant/anti-inflammatory herbal extract supplementation could represent an innovative approach to contrast IBDs. Clinical trials demonstrated the efficacy of natural formulas, containing chamomile, in patients with gastrointestinal disorders. This is consistent, albeit in part, with the antioxidant and anti-inflammatory properties of chamomile. The aim of the present study was to explore the possible protective role of a chamomile extract, on human colorectal adenocarcinoma HT29 cell, and rat colon specimens treated with lipopolysaccharide (LPS) to induce an inflammatory stimulus, a well established model of acute ulcerative colitis. In this context, the activities of different biomarkers of inflammation and lipid peroxidation such as ROS, myeloperoxidase (MPO), serotonin (5-HT), prostaglandin (PG)E_2, 8-iso-prostaglandin (8-iso-PG)$F_{2\alpha}$, NF-kB, tumor necrosis factor (TNF)α and interleukin (IL)-6 were assessed. We found that chamomile extract was as effective as sulfasalazine (2 mg/ml) in reducing the production of MPO, 5-HT, IL-6, NF-kB, TNFα, PGE_2 and 8-iso-$PGF_{2\alpha}$, after inflammatory stimulus. The observed modulatory effects support a rationale use of chamomile supplementation as a promising pharmacological tool for the prevention and management of ulcerative colitis in humans. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: colon; immunomodulation; chamomile; inflammation; oxidative Stress.

INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic disorders characterized by disruption and ulceration of the colonic mucosa (ulcerative colitis) or of any part of the digestive tract (Crohn's disease). Although IBDs etiology is still a matter of debate, oxidative stress seems to play a pivotal role (Koutroubakis *et al*., 2004; Rezaie *et al*., 2007; Achitei *et al*., 2013). On the other hand, antioxidant/anti-inflammatory herbal extract supplementation could represent an innovative approach to contrast IBDs symptoms (Chung *et al*., 2007; Lenoir *et al*., 2012).

Chamomile has long been used as a medicinal plant in the management of gastrointestinal disorders (McKay and Blumberg, 2006). The rationale for the traditional use has been recently corroborated by multiple clinical trials (Langhorst *et al*., 2013; Albrecht *et al*., 2014). This is consistent, albeit in part, with the antioxidant and anti-inflammatory properties of chamomile (McKay and Blumberg, 2006; Drummond *et al*., 2013).

The aim of the present study was to explore the possible protective role of chamomile on human colorectal adenocarcinoma HT29 cell line, and isolated rat colon specimens treated with lipopolysaccharide (LPS) to induce an inflammatory stimulus, a well established model of acute ulcerative colitis. (Bahar *et al*., 2012). In this context, the activities of different biomarkers of colon inflammation and lipid peroxidation such as reactive oxygen species (ROS), myeloperoxidase (MPO), serotonin (5-HT), prostaglandin (PG)E_2, 8-iso-prostaglandin (8-iso-PG)$F_{2\alpha}$ were assessed (Nagib *et al*., 2013; Motavallian et al., 2013; Regmi *et al*., 2014). Finally, we evaluated the immune response modulatory effects of chamomile, by measuring the mRNA levels of cytokines playing a key role in colon epithelium damage, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), tumor necrosis factor (TNF)α and interleukin (IL)-6 (Feghali and Wright, 1997; Lee *et al*., 2010).

MATERIAL AND METHODS

Plant extract. Chamomile extract were kindly furnished by Aboca S.p.A. It consists of freeze-dried extract obtained from ligulate flowers collected from cultivated plants of *Chamomilla recutita* (L.) Rauschert. Solvent, temperature, plant-solvent weight ratio and extraction process are optimized for active principles recovery and stability, and the resulting extract is characterized by high flavonoid content. Origin of the extract were guarantee by producer and qualitative standard were defined as total apigenin content, total phenols and total flavonoids. Phytochemical composition of final extracts is described in Table 1. The extract, consisting in a dry

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Table 1. Phytochemical composition of the tested chamomile extract

Total apigenin (as sum of apigenin, apigenin-7-glucoside and apigenin-7-(6-acetil)glucoside)	4.52 ± 0.28%
Total phenols (as mg of gallic acid equivalents per g of extract)	56.62 ± 1.31 mg/g d.e.
Total flavonoids (as mg of rutin equivalents per g of extract)	21.53 ± 0.86 mg/g d.e.

pale yellowish powder, was stored at −20 °C and solubilized immediately before test.

In vitro* studies.** ***Cell culture and viability test. HT29 cells were cultured in DMEM (Euroclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1.2% (v/v) penicillin G/streptomycin in 75 cm^2 tissue culture flask ($N = 5$ individual culture flasks for each condition). The cultured cells were maintained in humidified incubator with 5% CO_2 at 37 °C. For cell differentiation, HT29 cell suspensions at a density of 1×10^6 cells/mL were treated with various concentrations (10, 50, and 100 ng/mL) of phorbol myristate acetate (PMA, Fluka) for 48 h (induction phase). Thereafter, the PMA-treated cells were washed twice with ice-cold pH 7.4 phosphate buffer solution (PBS) to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). Morphology of cells was examined under an inverted phase-contrast microscope (Sintiprungrat *et al*., 2010). To assess the basal cytotoxicity of chamomile, a viability test was performed on 96 microwell plates, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) test. Macrophages were incubated with extracts (ranging concentration 10–1000 μg/mL) for 24 h. About 10 μL of MTT (5 mg/mL) was added to each well and incubated for 3 h. The formazan dye formed was extracted with dimethyl sulfoxide and absorbance recorded as previously described (Menghini *et al*., 2011). Effects on cell viability were evaluated in comparison to untreated control group.

Reactive oxygen species generation. Reactive oxygen species generation was assessed using a ROS-sensitive fluorescence indicator, DCFH-DA. When DCFH-DA is introduced to viable cells, it can penetrate the cell and become deacetylated by intracellular esterases to form 2′,7′-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell, and be converted to 2′,7′-dichlorofluorescein (DCF), which is detected by a fluorescence spectrophotometer. To determine intracellular effects on ROS production, cells were seeded in a black 96-well plate (1.5 × 104 cells/well) in medium containing scalar concentration of extracts. Immediately after seeding, the cells were stimulated for 1 h with H_2O_2 (1 mM). After the cells were incubated with DCFH-DA (20 μM) for 30 min, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a fluorescence microplate reader.

***Ex vivo* studies.** Twenty-four male adult Sprague–Dawley rats (200–250 g) were housed in plexiglas cages (40 cm × 25 cm × 15 cm), one rat per cage, in climatized colony rooms (22 ± 1 °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research. According to the recognized ethical principles of 'Replacement, Refinement and Reduction of Animals in Research', colon specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Local Ethical Committee (G. d'Annunzio University) and Italian Health Ministry.

Rats were sacrificed by CO_2 inhalation (100% CO_2 at a flow rate of 20% of the chamber volume per minute) and colon specimens were immediately collected and maintained in humidified incubator with 5% CO_2 at 37 °C for 4 h, in DMEM buffer with added bacterial LPS (10 μg/mL) (incubation period). During the incubation period, tissues were treated with scalar sub-toxic concentrations of chamomile extract (100–1000 μg/mL). The efficacy of chamomile extract was evaluated in comparison with sulfasalazine (2 mg/mL), that is used as reference drug whose efficacy could be partially related to reduced activity of cyclooxygenase and lipoxygenase. Tissue perfusates were collected and PGE_2 and 8-iso-$PGF_{2\alpha}$ levels (ng/mg wet tissue) were measured by radioimmunoassay (RIA), as previously reported (Chiavaroli *et al*., 2010; Menghini *et al*., 2010). On the other hand, individual colon specimens were dissected and subjected to extractive procedures to evaluate MPO activity (mU/mg wet tissue), 5-HT steady state level (ng/mg wet tissue), NF-kB, IL-6 and TNFα gene expression, as previously reported (Krawisz *et al*., 1984; Brunetti *et al*., 2013).

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Means ± S.E.M. were determined for each experimental group and analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls comparison multiple test. Statistical significance was set at $p < 0.05$. As regards to gene expression analysis, the comparative $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and then determine the relative changes in individual gene expression (relative quantification) (Livak and Schmittgen, 2001). Finally, as regards to the animals randomized for each experimental group, the number was calculated on the basis of the 'Resource Equation' $N = (E + T)/T$ $(10 \leq E \leq 20)$ elaborated by the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3RS) and reported on the following web site: https://www.nc3rs.org.uk/experimental-designstatistics.

RESULTS

Our *in vitro* study showed that chamomile extract was well tolerated by HT29 cell line in the range (10–1000 μg/mL) (Fig. 1). Moreover, we observed a protective effect exerted by chamomile extract

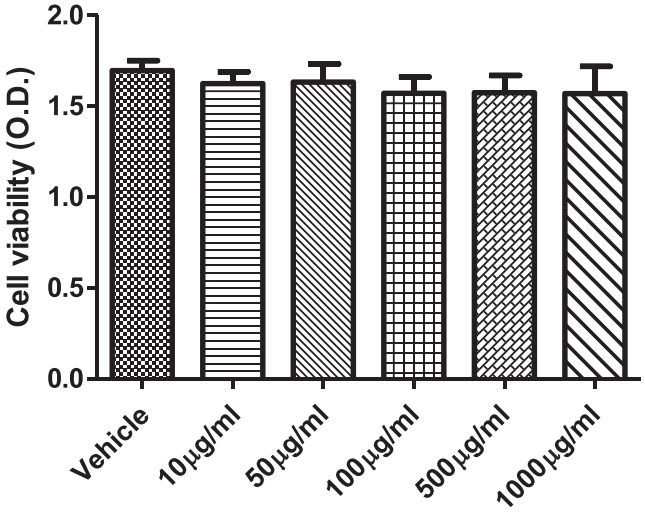

Figure 1. Effect of chamomile extract (10–1000 μg/mL) on HT29 cell line viability.

(10–1000 μg/mL) as revealed by the significant reduction of H_2O_2-induced (1 mM) ROS production (Fig. 1).

The preliminary *in vitro* test revealed a valuable index of non-toxic and effective concentrations to define the concentration for colon tissue treatment. In the *ex vivo* experiments, in colon tissues exposed to LPS-induced inflammatory stimulus, we found that chamomile extract (100–1000 μg/mL) was effective in reducing the oxidative stress, inflammation and immune response biomarkers, such as MPO, 5-HT, NF-kB, IL-6, TNFα, PGE_2 and 8-iso-$PGF_{2\alpha}$ (Figs. 2–9). The efficacy was comparable to sulfasalazine (2 mg/mL).

DISCUSSION

Oxidative stress is an imbalance in the pro-oxidant/antioxidant homeostasis, characterized by overproduction of reactive oxygen/nitrogen species (ROS/RNS) that could drive to disruptive peroxidation reactions on

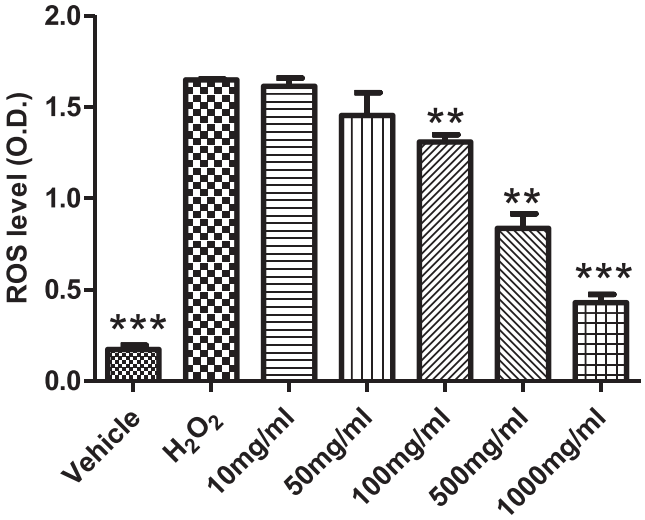

Figure 2. Effect of chamomile extract (10–1000 μg/mL) on H_2O_2 reactive oxygen species (ROS) production in HT29 cell line. ANOVA, $p < 0.0001$, post hoc $^{**}p < 0.01$, $^{***}p < 0.001$ vs H_2O_2 group.

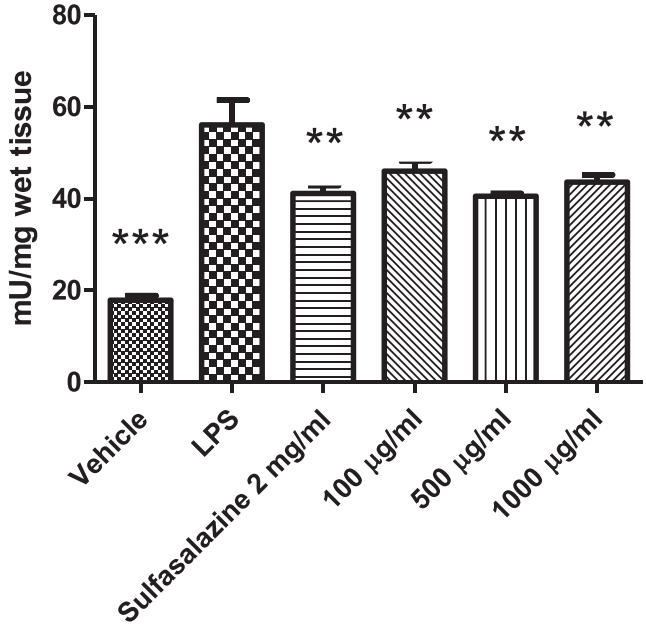

Figure 3. Effect of chamomile extract (100–1000 μg/mL) on myeloperoxidase activity (mU/ng wet tissue). ANOVA, $p < 0.0001$, post hoc $^{**}p < 0.01$, $^{**}p < 0.001$ vs LPS-treated group.

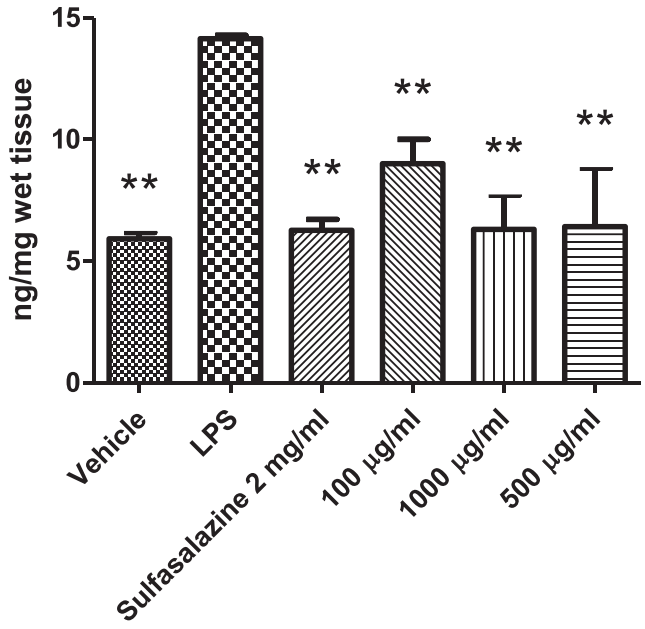

Figure 4. Effect of chamomile extract (100–1000 μg/mL) on prostaglandin E2 (PGE_2) levels. ANOVA, $p < 0.01$, post hoc $^{**}p < 0.01$ vs LPS-treated group.

cellular substrates such as proteins, lipids, and nucleic acids (Uttara *et al.*, 2009). In particular, lipid peroxidation has been recognized as a crucial step in the pathogenesis of several disease states, including IBDs (Achitei *et al.*, 2013). ROS and RNS are mainly produced by macrophages and neutrophils, and the effects of these reactive species include neutrophil recruitment at the inflamed epithelial colon tissue. 8-iso-$PGF_{2\alpha}$, deriving from ROS/RNS peroxidation of membrane arachidonic acid, represents a stable marker of oxidative stress, *in vivo* (Praticò, 2002), and our experiments demonstrate that 8-iso-$PGF_{2\alpha}$ production is reduced in inflamed rat colon (Fig. 4) in concentration-dependent

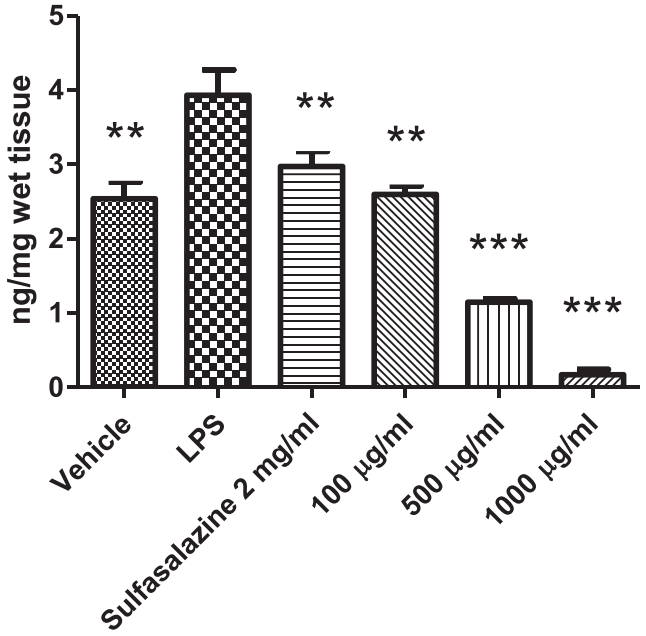

Figure 5. Effect of chamomile extract (100–1000 μg/mL) on 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels (ng/mg wet tissue). ANOVA, $p < 0.0001$, post hoc $^{**}p < 0.01$, $^{***}p < 0.001$ vs LPS-treated group.

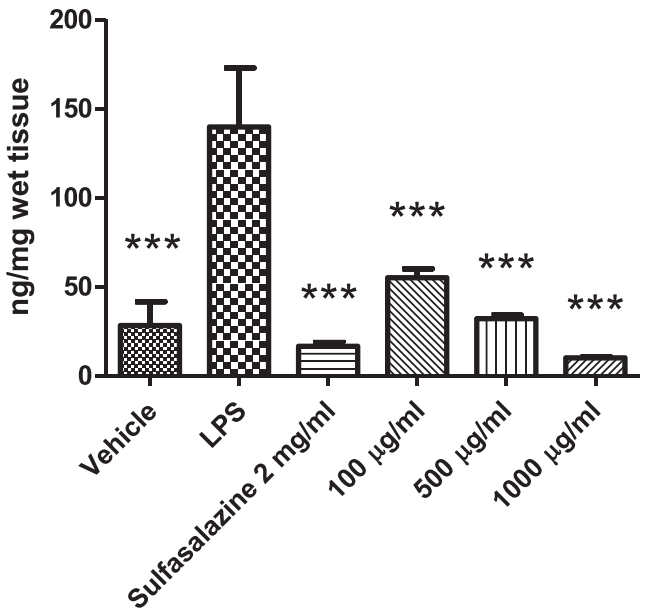

Figure 6. Effect of chamomile extract (100–1000 μg/mL) on serotonin (5-HT) levels (ng/mg wet tissue). ANOVA, $p < 0.01$, post hoc $^{***}p < 0.001$ vs LPS-treated group.

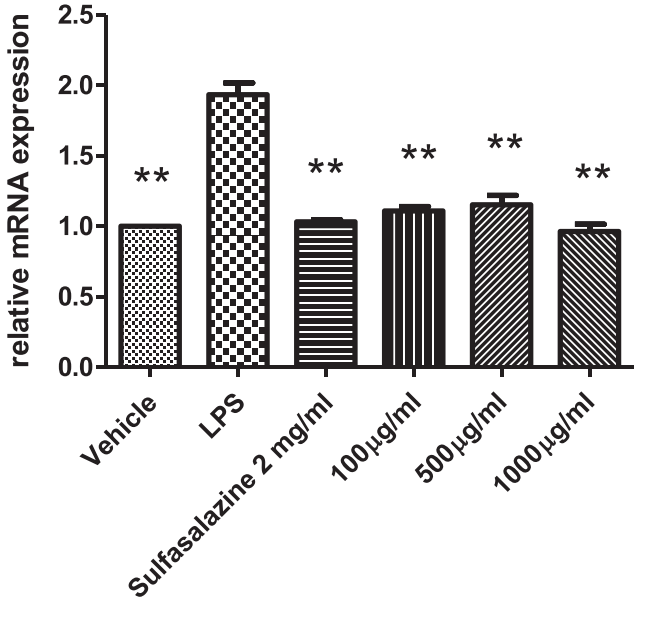

Figure 7. Effect of chamomile extract (100–1000 μg/mL) on tumor necrosis factor α (TNFα) gene expression. ANOVA, $p < 0.0001$, post hoc $^{**}p < 0.01$, $^{***}p < 0.001$ vs respective LPS-treated group.

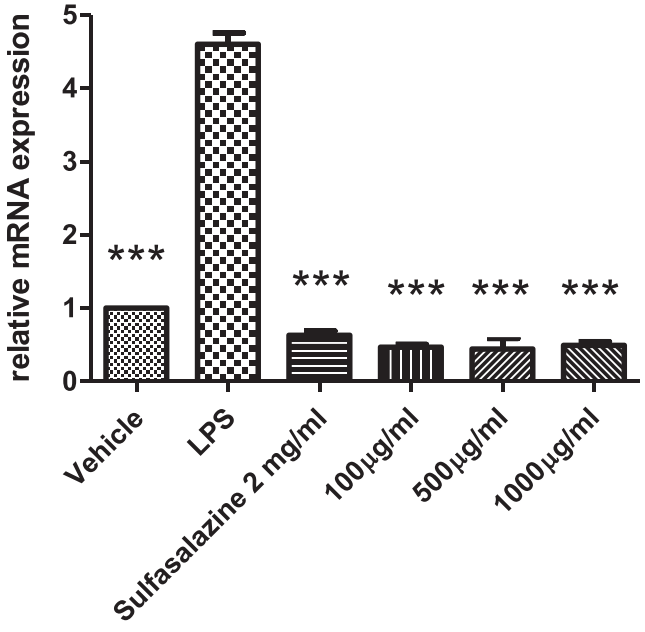

Figure 8. Effect of chamomile extract (100-1000 μg/mL) on interleukin-6 (IL-6) gene expression. ANOVA, $p < 0.0001$, post hoc $^{***}p < 0.001$ vs respective LPS-treated group.

manner, after exposure to chamomile extracts. This could partially derive by the concentration-dependent radical scavenging activity of chamomile (Lee and Shibamoto, 2002), that was confirmed by the observed concentration dependent reduction of ROS on HT29 cell line exposed to H_2O_2 (1 mM)-induced oxidative stress (Fig. 2), and could explain the observed inhibitory effect on MPO activity (Fig. 3), a biomarker of neutrophil infiltration (Talero *et al.*, 2007). We also found a significant reduction in PGE_2 levels in the chamomile-treated colons (Fig. 4). This reduction could be, albeit partially, related to a possible inhibitory effect on cyclo-oxygenase (COX) 2 activity, as previously suggested by Srivastava and colleagues (Srivastava *et al.*, 2009). Finally, we tested the modulatory effects of chamomile on colonic TNFα, IL-6 and 5-HT production. 5-HT pro-inflammatory role in IBDs has been previously suggested (Regmi *et al.*, 2014), possibly involving the activation of 5-HT3 receptors (Mousavizadeh *et al.*, 2009). Our results showed that chamomile extracts are able to reduce colonic 5-HT levels (Fig. 6). Multiple comparative studies confirmed that neurotransmitter steady state level is a valuable index of neurotransmitter release *in vivo* (Brunetti *et al.*, 2014). Actually, our findings of reduced colon 5-HT levels induced by chamomile extract is consistent with the concentration-

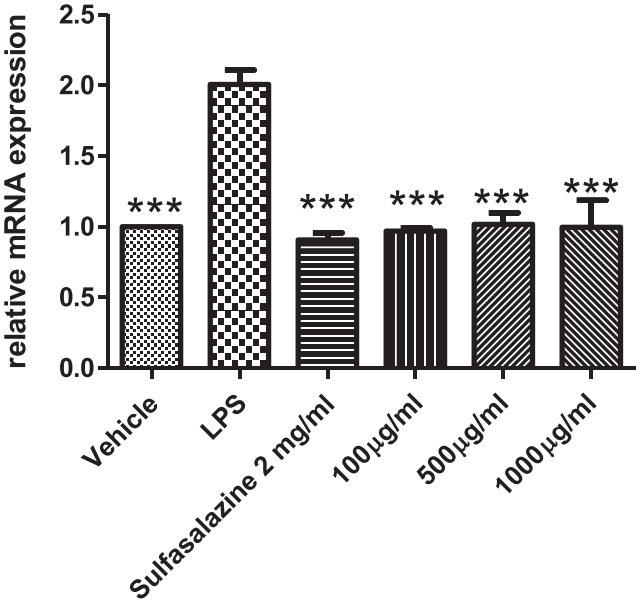

Figure 9. Effect of chamomile extract (100–1000 µg/mL) on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) gene expression. ANOVA, $p < 0.0001$, post hoc $^{***}p < 0.001$ vs respective LPS-treated group.

dependent apigenin-induced inhibition of gut 5-HT release, *in vitro* (Zhao *et al*., 2010). In ulcerative colitis, the infiltration of intestinal mucosa by macrophages and neutrophils also enhances the local levels of pro-inflammatory cytokines, such as TNFα and IL-6, which are known to play a key role in mediating tissue damage (Bouguen *et al*., 2011). Finally, we found that chamomile extract reduce the NF-kB gene expression induced by LPS pre-treatment in colon specimens. Considering that NF-kB is a transcriptor factor involved in the expression of cytokines and inflammatory enzymes such as iNOS, COX2 and MPO (Piva *et al*., 2006; Hartman *et al*., 2011; Rashidian *et al*., 2016), we cannot exclude that the concentration independent inhibitory effect observed for NF-kB gene expression, could be a common inhibitory pathway for the observed MPO, TNFα and PGE_2 activities, following exposition to chamomile extract. In this context, we have investigated the possible immune-modulatory effects of chamomile extracts, finding a significant inhibition of both basal and LPS-induced TNFα and IL-6 activity in colon specimens, as revealed by the reduction of their mRNA levels (Fig. 7, 8). These data corroborate the previous reported inhibitory effects induced by both apigenin and chamomile extracts on cytokine production, *in vitro* (McKay and Blumberg, 2006; Drummond *et al*., 2013).

The inhibitory effects exerted by chamomile extract on the tested biomarkers are consistent with the observed positive clinical effects induced by chamomile herbal formulations on IBDs symptoms (Langhorst *et al*., 2013; Albrecht *et al*., 2014). On the other hand, recent papers suggested that anti-inflammatory modulatory effects could be related, albeit partially, to the terpenoid fractions, particularly α(-)bisabolol, bisabolol oxide A, and guaiazulene (Maurya *et al*., 2014; Ortiz *et al*., 2016).

In conclusion, in the present work, we have investigated the possible efficacy of a commercial chamomile extract in modulating the inflammatory and immune response, in an *ex vivo* experimental model of IBD (Bahar *et al*., 2012). The observed modulatory effects support a rationale use of chamomile supplementation as a promising pharmacological tool for the prevention and management of ulcerative colitis in humans. On the other hand, the immunomodulatory effects of the extract need to be evaluated in experimental *in vivo* models. Considering that the *ex vivo* evaluation of chamomille extract has its own inherent limitations, further investigations, comparing different analytical methods and experimental paradigms, including *in vivo* studies, for detection and quantification of oxidative stress, inflammation and immune response biomarkers are required for an accurate evaluation of chamomile efficacy.

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This work is dedicated to the loving memory of Professor Giovanni Ciabattoni (1951-2014), man of science, culture and humanity, to whom the authors would once again express their gratitude for his precious and priceless teachings.

Conflict of Interest

Authors declare no financial/commercial conflicts of interest.

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