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

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Original
An Hydroalcoholic Chamomile Extract Modulates Inflammatory and Immune Response in HT29 Cells and Isolated Rat Colon / Menghini, L., Ferrante, M.C., Leporini, L., Recinella, L., Chiavaroli, A., Leone, S., Pintore, G., Vacca, M., Orlando, G., Brunetti, L.. - In: PHYTOTHERAPY RESEARCH. - ISSN 0951-418X. - 30:9(2016), pp. 1513-1518. [10.1002/ptr.5655]

Availability:
This version is available at: 11388/224497 since: 2022-06-07T11:02:36Z

Publisher:

Published
DOI:10.1002/ptr.5655

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(Article begins on next page)

19 November 2026



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Journal:	*Phytotherapy Research*
Manuscript ID	PTR-16-0318
Wiley - Manuscript type:	Full Paper
Date Submitted by the Author:	09-Mar-2016
Complete List of Authors:	Menghini, Luigi; University "G. d'Annunzio" of Chieti-Pescara, Department of Pharmacy Ferrante, Claudio; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy Leporini, Lidia; University "G. d'Annunzio" of Chieti-Pescara, Department of Pharmacy Recinella, Lucia; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy Chiavaroli, Annalisa; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy Leone, Sheila; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy PINTORE, GIORGIO; university, chemistry and paharmacy Vacca, Michele; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy Orlando, Giustino; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy Brunetti, Luigi; University "G. d\'Annunzio" of Chieti-Pescara, Department of Pharmacy
Keyword:	Colon, Immunomodulation, Chamomile, Inflammation, Oxidative Stress

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AN IDROALCHOLIC CHAMOMILE EXTRACT MODULATES INFLAMMATORY AND IMMUNE RESPONSE IN ISOLATED RAT COLON CHALLENGED WITH LPS

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Key words: Colon, Immunomodulation, Chamomile, Inflammation, Oxidative Stress

Chamomile modulate colonic inflammation

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ABSTRACT

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 line, and isolated 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)$F_{2\alpha}$, tumor necrosis factor (TNF)α and interleukin (IL)-6 were assessed. We found that chamomile extract was as effective as sulfasalazine (5 mM) in reducing the production of MPO, 5-HT, IL-6, 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.

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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 (Rezaie et al., 2007; Achitei et al., 2013; Koutroubakis 2004). 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 et al, 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 et al, 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 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 tumor necrosis factor (TNF)α and interleukin (IL)-6 (Feghali et al., 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. Phytochemical composition of final extracts is described in table I.

(Please insert table I)

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 doses (10, 50, and 100 ng/ml) of phorbol myristate acetate (PMA, Fluka) for 24 h or 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

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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.

ROS generation

ROS 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, synaptosomes were seeded in a black 96-well plate (1.5 x 104 cells/well) in medium containing scalar concentration of extracts. Immediately after seeding, the synaptosomes 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

24 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 (5 mM). 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), IL-6 and TNFα gene expression, as previously reported (Krawisz et al., 1984; Brunetti et al., 2013).

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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 et al., 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≤E≤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 HT29cell line in the range (10-1000 µg/ml) (Fig.1). Moreover, we observed a dose-dependent protective effect exerted by chamomile extract (10-1000 µg/ml) as revealed by the significant reduction of H_2O_2-induced (1 mM) ROS production (Fig.1).

(Please insert Figs.1-2)

The preliminary *in vitro* test revealed a valuable index of non-toxic and effective doses 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, IL-6, TNFα, PGE_2 and 8-iso-$PGF_{2\alpha}$ (Figs. 3-8). The efficacy was comparable to sulfasalazine (5 mM).

(Please insert Figs. 3-8)

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 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 et al., 2002), and our experiments demonstrate that 8-iso-$PGF_{2\alpha}$ production is reduced in inflamed rat colon (Fig.4), after administration of chamomile extracts. This could partially derive by the radical scavenging activity of chamomile (Lee et al., 2002), that was confirmed by our evaluations 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

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chamomile-treated colons (Fig.4). This reduction could be, albeit partially, related to a possible inhibitory effect on cyclooxygenase 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 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 (Bounguen et al., 2011). 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 et al., 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).

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. Since each technique measures something different and has its own inherent limitations, further investigations, comparing different analytical methods and experimental paradigms for detection and quantification of oxidative stress, inflammation and immune response biomarkers are required for an accurate evaluation of chamomile efficacy.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian Ministry of University.

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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Figure 1

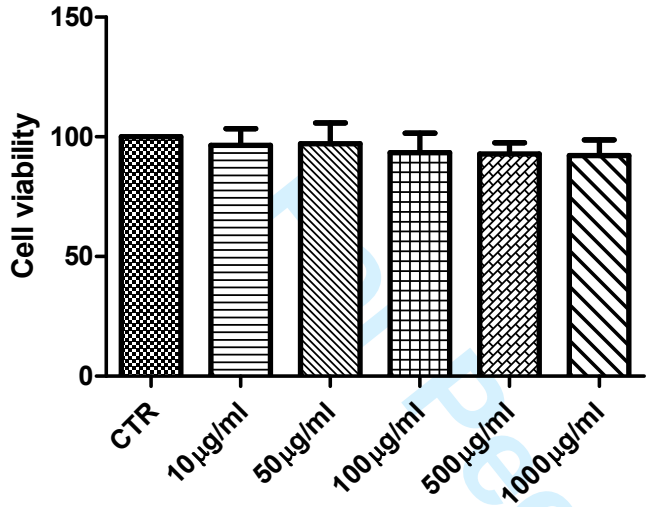
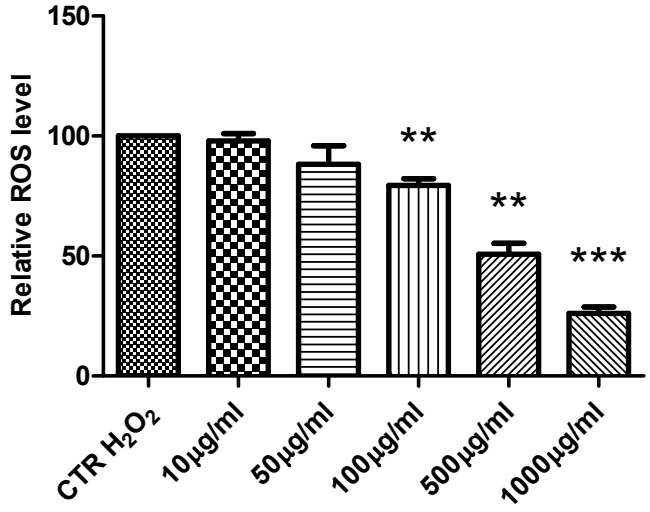


Figure 1: Effect of chamomile extract (10-1000 µg/ml) on HT29 cell line viability.

Figure 2



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Figure 2: : Effect of chamomile extract (10-1000 µg/ml) on H_2O_2 ROS production in HT29 cell line. ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs H_2O_2-treated group

Figure 3

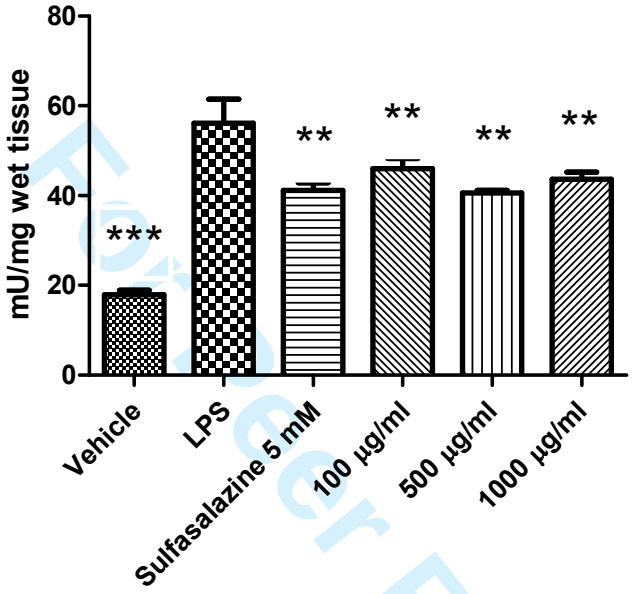


Figure 3: Effect of chamomile extract (100-1000 µg/ml) on mieloperossidase (MPO) activity (mU/ng wet tissue). ANOVA, P<0.0001, post hoc **P<0.01, **P<0.001 vs LPS-treated group.

Figure 4

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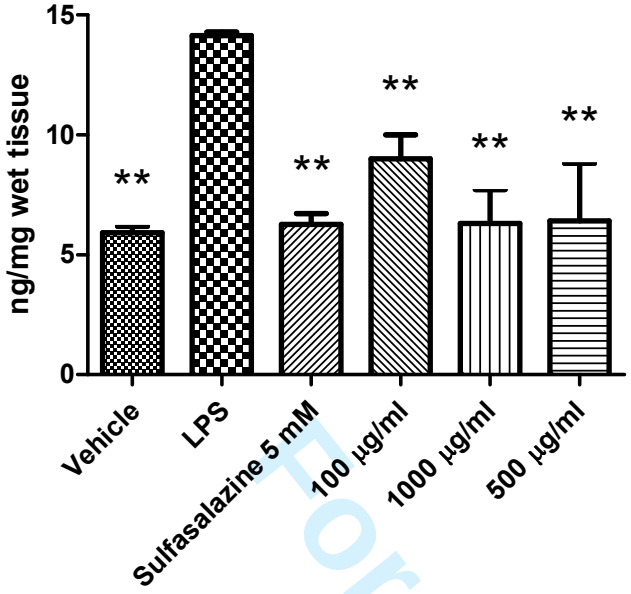


Figure 4: Effect of chamomile extract (100-1000 µg/ml) on prostaglandin E2 (PGE_2) levels. ANOVA, P<0.01, post hoc **P<0.01 vs LPS-treated group.

Figure 5

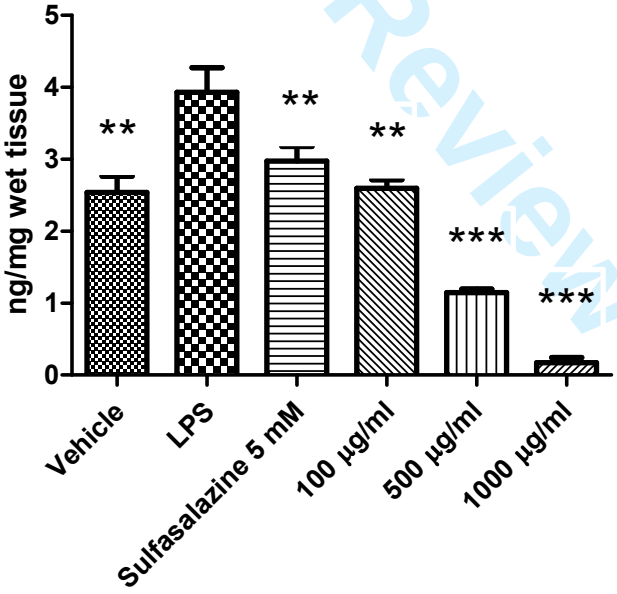


Figure 5: Effect of chamomile extract (100-1000 µg/ml) on 8-iso-prostaglandin F2α (8-iso-$PGF_{2\alpha}$) levels (ng/mg wet tissue). ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs LPS-treated group.

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Figure 6

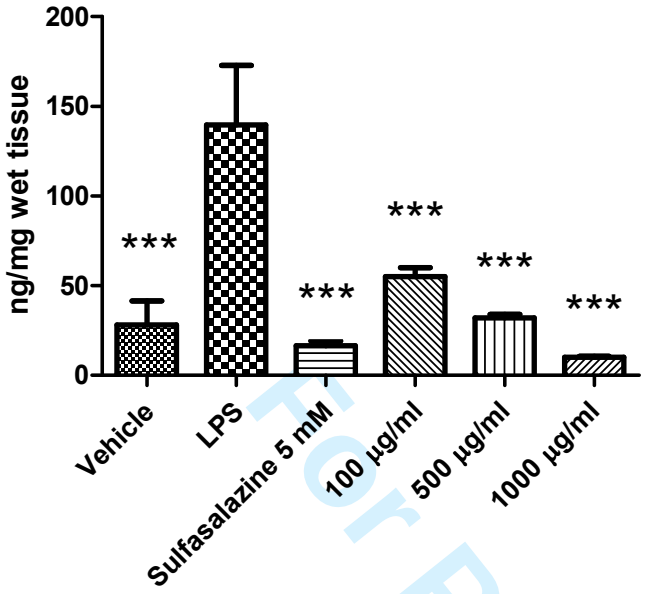


Figure 6: Effect of chamomile extract (100-1000 µg/ml) on serotonin (5-HT) levels (ng/mg wet tissue). ANOVA, P<0.01, post hoc ***P<0.001 vs LPS-treated group.

Figure 7

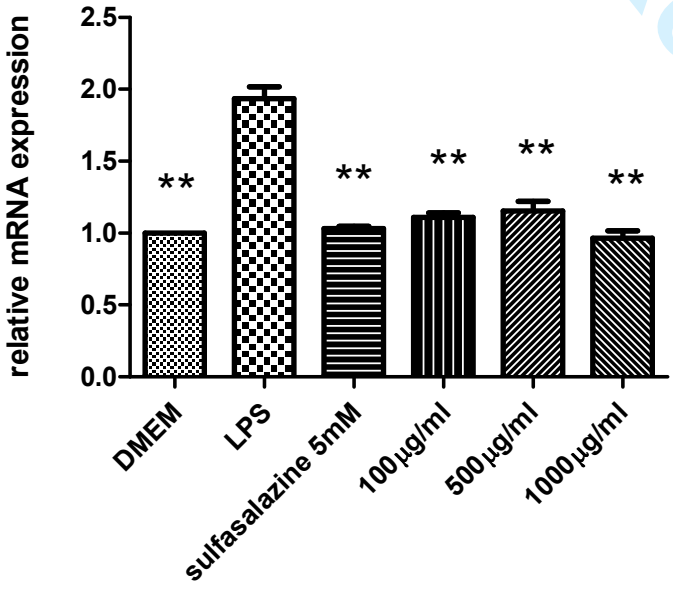


Figure 7: Effect of chamomile extract (100-1000 µg/ml) on tumor necrosis factor α (TNFα) and gene expression. ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs respective LPS-treated group.

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Figure 8

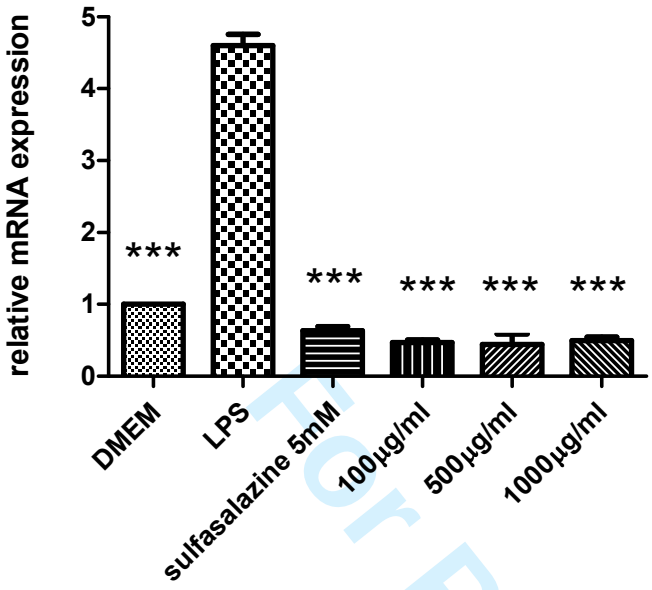


Figure 8: Effect of chamomile extract (100-1000 µg/ml) on interleukin-6 (IL-6) and gene expression. ANOVA, P<0.0001, post hoc ***P<0.001 vs respective LPS-treated group.

Table I: Phytochemical composition of the tested chamomile extract.

Table I	
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.

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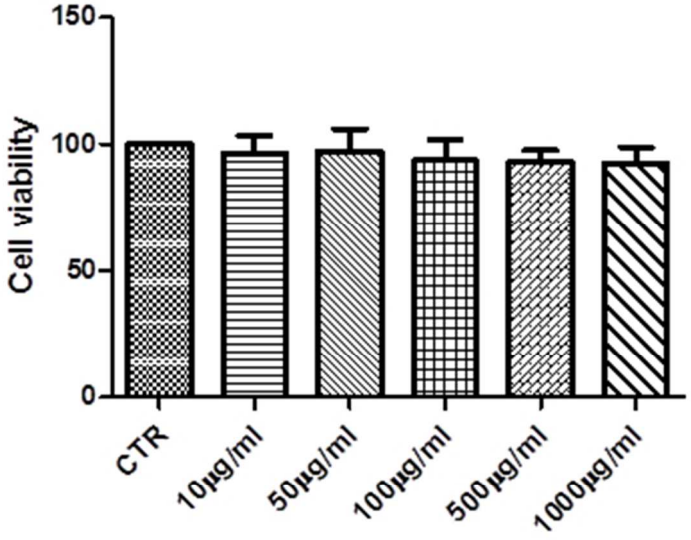


Figure 1: Effect of chamomile extract (10-1000 µg/ml) on HT29 cell line viability.
128x115mm (72 x 72 DPI)

For Review

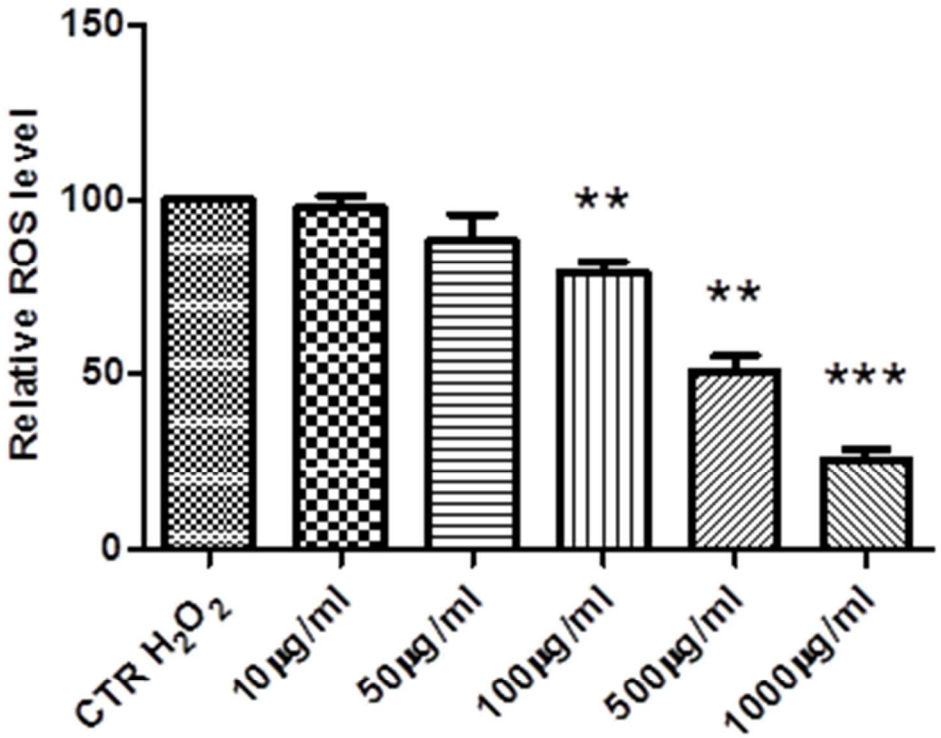


Figure 2: : Effect of chamomile extract (10-1000 µg/ml) on H2O2 ROS production in HT29 cell line. ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs H2O2-treated group
128x106mm (300 x 300 DPI)

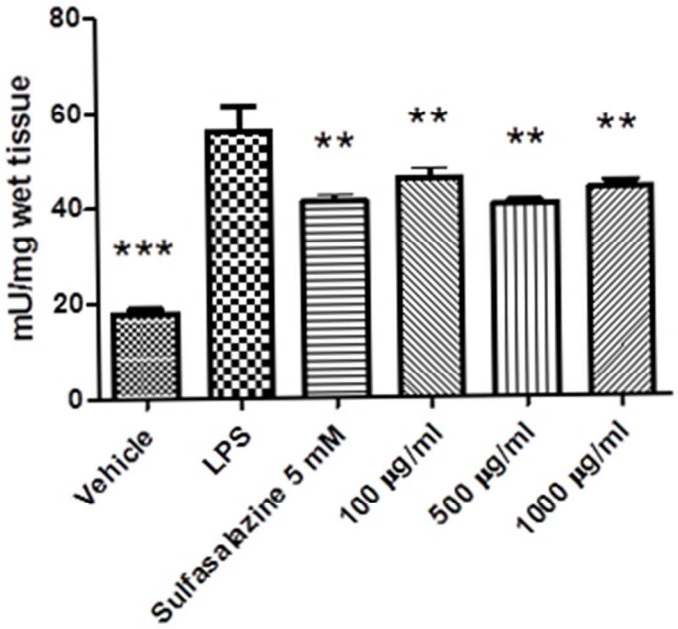


Figure 3: Effect of chamomile extract (100-1000 µg/ml) on mieloperossidase (MPO) activity (mU/ng wet tissue). ANOVA, P<0.0001, post hoc **P<0.01, **P<0.001 vs LPS-treated group.
31x27mm (300 x 300 DPI)

Review

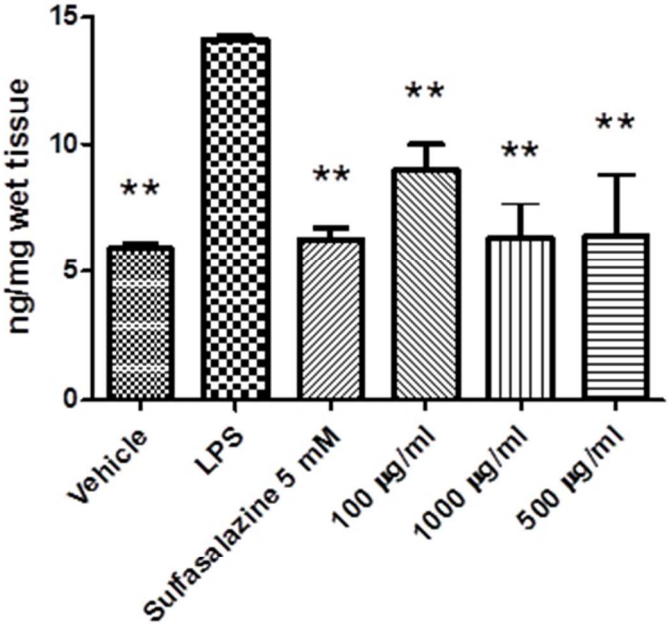


Figure 4. Effect of chamomile extract (100-1000 µg/ml) on prostaglandin E2 (PGE2) levels. ANOVA, P<0.01, post hoc **P<0.01 vs LPS-treated group.
30x31mm (300 x 300 DPI)

For Review

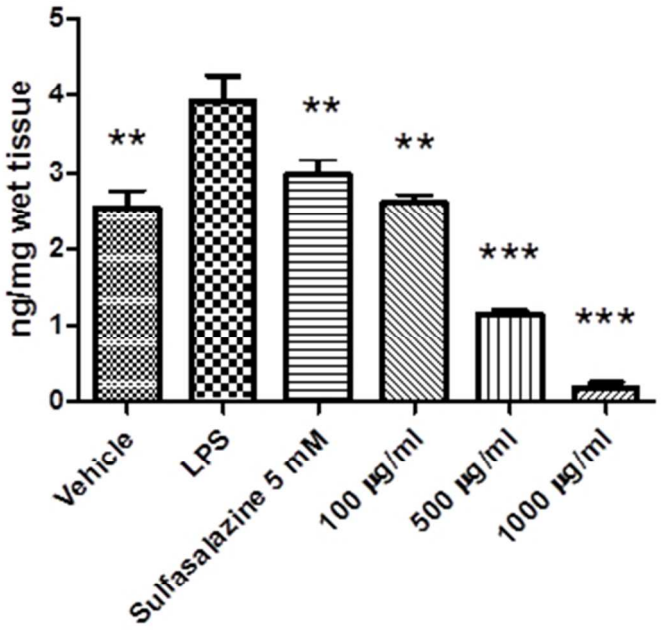


Figure 5: Effect of chamomile extract (100-1000 µg/ml) on 8-iso-prostaglandin F2α (8-iso-PGF2α) levels (ng/mg wet tissue). ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs LPS-treated group.
30x31mm (300 x 300 DPI)

For Review

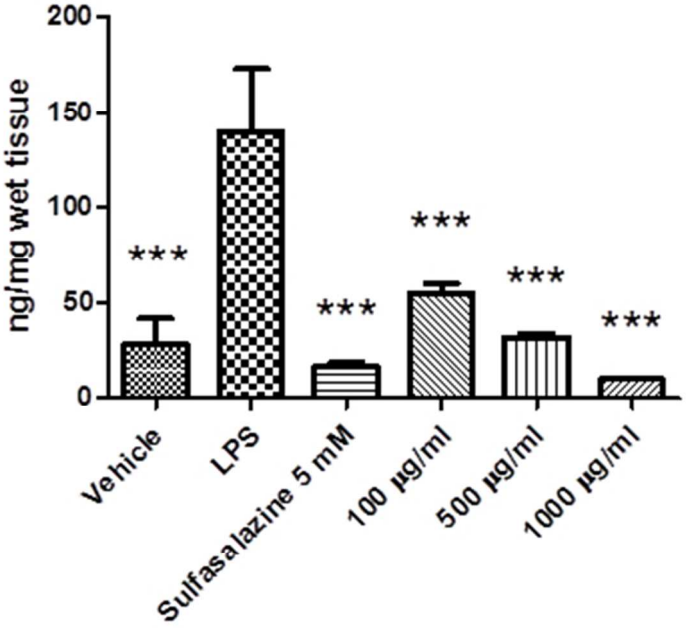


Figure 6: Effect of chamomile extract (100-1000 µg/ml) on serotonin (5-HT) levels (ng/mg wet tissue). ANOVA, P<0.01, post hoc ***P<0.001 vs LPS-treated group.
30x31mm (300 x 300 DPI)

For Review

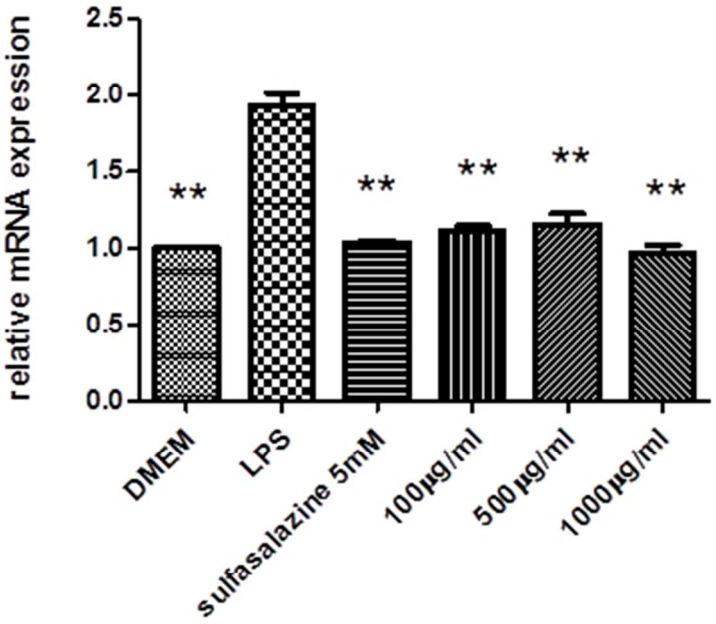


Figure 7: Effect of chamomile extract (100-1000 µg/ml) on tumor necrosis factor α (TNFα) and gene expression. ANOVA, P<0.0001, post hoc **P<0.01, ***P<0.001 vs respective LPS-treated group.
32x30mm (300 x 300 DPI)

For Review

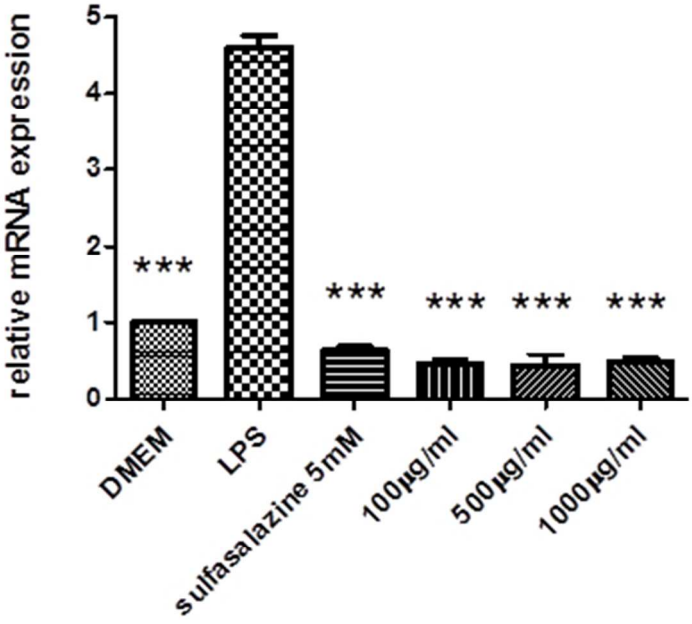


Figure 8: Effect of chamomile extract (100-1000 µg/ml) on interleukin-6 (IL-6) and gene expression.
ANOVA, P<0.0001, post hoc ***P<0.001 vs respective LPS-treated group.
31x30mm (300 x 300 DPI)

Review