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Original article

The anti-inflammatory and antioxidant effects of bergamot juice extract (BJe) in an experimental model of inflammatory bowel disease

Daniela Impellizzeri [a], Giuseppe Bruschetta [a], Rosanna Di Paola [a], Akbar Ahmad [a], Michela Campolo [a], Salvatore Cuzzocrea [a, b, *], Emanuela Esposito [a], Michele Navarra [c]

[a] Department of Biological and Environmental Sciences, University of Messina, Viale Ferdinando Stagno D'Alcontres, 31-98166 Messina, Italy
[b] Manchester Biomedical Research Centre, Manchester Royal Infirmary, School of Medicine, University of Manchester, UK
[c] Department of Drug Sciences and Products for Health, University of Messina, SS. Annunziata, Messina 98168, Italy

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SUMMARY

Background & aims: The beneficial properties of the flavonoid fraction of bergamot juice (BJe) have been raising interest and have been the subject of recent studies, considering the potentiality of its health promoting substances. Flavonoids have demonstrated radical-scavenging and anti-inflammatory activities. The aim of the present study was to examine the effects of BJe in mice subjected to experimental colitis.

Methods: Colitis was induced in mice by intracolonic instillation of dinitrobenzene sulfonic acid (DNBS). BJe was administered daily orally (at 5, 10 and 20 mg/kg).

Results: Four days after DNBS administration, colon nuclear factor NF-κB translocation and MAP kinase phospho-JNK activation were increased as well as cytokine production such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Neutrophil infiltration, by myeloperoxidase (MPO) activity, in the mucosa was associated with up-regulation of adhesion molecules (ICAM-1 and P-selectin). Immunohistochemistry for nitrotyrosine and poly ADP-ribose (PAR) also showed an intense staining in the inflamed colon. Treatment with BJe decreased the appearance of diarrhea and body weight loss. This was associated with a reduction in colonic MPO activity. BJe reduced nuclear NF-κB translocation, p-JNK activation, the pro-inflammatory cytokines release, the appearance of nitrotyrosine and PAR in the colon and reduced the up-regulation of ICAM-1 and P-selectin. In addition, colon inflammation was also associated with apoptotic damage. Treatment with BJe caused a decrease of pro-apoptotic Bax expression and an increase of anti-apoptotic Bcl-2 expression.

Conclusions: The results of this study suggested that administration of BJe induced, partly specified, anti-inflammatory mechanisms, which potentially may be beneficial for the treatment of IBD in humans.

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1. Introduction

Crohn's disease (CD) and ulcerative colitis are common inflammatory bowel diseases (IBD). These are characterized by severe inflammation with abnormal cytokine production, augmentation in adhesion molecule expression and cell infiltrate, which ultimately lead to epithelial cell apoptosis and mucosal damage. Oxidative stress also plays a significant role in the pathogenesis of IBD, including CD. Endogenous antioxidants such as superoxide dismutase (SOD), glutathione and catalase are normally able to counteract oxidative stress in the intestinal mucosa [1]. However, inflammation increases the demand for these antioxidants and results in an imbalance between pro-oxidants and antioxidants with subsequent mucosal damage [1]. Several animal models of IBD have been developed which show similarities to human CD although most models do not predict what happens in human immunology. Between these, the model of colonic inflammation induced by dinitrobenzene sulfonic acid (DNBS) delivered intrarectally to normal mice displays human CD features, notably

Abbreviations: CD, Crohn's disease; BJe, bergamot juice; DNBS, dinitrobenzene sulfonic acid; NF-κB, nuclear factor; TNF-α, tumor necrosis factor; IL-1β, interleukin; MPO, myeloperoxidase; PAR, poly ADP-ribose; IBDs, inflammatory bowel diseases; ROS, reactive oxygen species.

* Corresponding author. Department of Biological and Environmental Sciences, University of Messina, Viale Ferdinando Stagno D'Alcontres, 31-98166 Messina, Italy. Tel.: +39 90 6765208.
E-mail address: salvator@unime.it (S. Cuzzocrea).

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predominant nuclear factor κB (NF-κB)-dependent CD4+ T-helper (Th1) activation [2].

Citrus bergamia Risso & Poiteau, a small tree belonging to the Rutaceae family, is cultivated almost exclusively along the southern coast of Calabria region (Italy), where the particular environmental conditions are favorable for its cultivation. Bergamot fruit is mostly used for the extraction of essential oil, widely used in perfume industry and recently investigated for its neuroprotective [3] and anticancer effects [4]. Bergamot juice (BJ), instead, obtained from the endocarp of the fruit, is considered just a secondary and discarded product. Different studies have analyzed the chemical composition of BJ [5–7] but few studies focused on its potential biological activities. Recently it has been shown that BJ reduced growth rate of different cancer cell lines, by mechanisms that in SH-SY5Y neuroblastoma cells are linked to an early impairment in cell adhesive and migratory machinery [7]. The anti-metastatic effect of BJ was also observed in a spontaneous metastatic neuroblastoma SCID mouse model [8]. Very recently it has been shown that low concentrations of BJ extract (BJe), the flavonoid fraction of BJ, reduces LPS-induced inflammatory response in THP-1 monocytes, through SIRT1-mediated NF-κB inhibition, laying the foundation for future investigations in *in vivo* animal models [9,10].

It is known that flavonoids may be effective in inflammatory disease and cancer [11] and are protecting in dermal damage [12]. Several mechanisms have been proposed, including the suppression of cyclooxygenase-2 (COX-2) expression [13], decrease of reactive oxygen species (ROS) [14], modulation of signaling pathways and down-regulation of NF-κB [15]. Thus, the objective of the present study was to demonstrate the anti-inflammatory and antioxidant effects of BJe on the animal model of colonic inflammation induced by DNBS in mice. In particular, the treatment with BJe decreased 1) histological damage, 2) NF-κB translocation, 3) neutrophil infiltration, 4) inflammatory cytokines levels (TNF-α and IL-1β), 5) adhesion molecules expression (ICAM-1 and P-selectin), 6) nitrotyrosine and PAR expression, 7) MAP kinase (phospho-JNK) expression and 8) apoptosis.

2. Materials and methods

2.1. "BJe" extract

BJe has been provided by the company "Agrumaria Corleone" (Palermo, Italy) which used fruits of *C. bergamia* Risso & Poiteau coming from crops located in the province of Reggio Calabria (Italy). BJe was centrifuged at 6000 rpm/min for 15 min to remove any impurities and successively transformed into a dry powder by the method of spray drying. Small aliquots of BJe were stored at −20 °C. Finally, the drug was defrosted and dissolved in non-pyrogenic saline just prior to use. The chemical composition of BJe has already been described [9].

2.2. Animals

Male adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light, 12-h dark cycle. The animals were acclimated to their environment for 1 week and had ad libitum access to tap water and rodent standard diet. The study was approved by the University of Messina Review Board for the care of animals. All animal experiments complied with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA).

2.3. Induction of experimental colitis

Colitis was induced with a very low dose of DNBS (4 mg per mouse) by using a modification of the method first described in rats [16]. In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by Enflurane. DNBS (4 mg in 100 μl of 50% ethanol) injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Vehicle alone (100 μl of 50% ethanol) was administered in control experiments (sham). Thereafter, the animals were kept for 15 min in a trendelenburg position to avoid reflux. After colitis and sham-colitis induction, the animals were observed for 4 days. On day 4, the animals were weighed and anaesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and biochemical studies.

2.4. Experimental groups

Animals were randomly divided into several groups ($n = 10$ for each group):

1. Sham + vehicle group: vehicle solution (saline) was administered by oral gavage for 4 days.
2. Sham + BJe (5 mg/kg): was administered by oral gavage for 4 days.
3. Sham + BJe (10 mg/kg): was administered by oral gavage for 4 days.
4. Sham + BJe (20 mg/kg): was administered by oral gavage for 4 days.
5. DNBS + vehicle: Mice were subjected to DNBS administration described as above, and vehicle (saline) was administered by oral gavage every 24 h, for 4 days, starting from 1 h after the administration of DNBS.
6. DNBS + BJe (5 mg/kg): Mice were subjected to DNBS administration described as above, and BJe (5 mg/kg) was administered by oral gavage every 24 h, for 4 days starting from 1 h after the administration of DNBS.
7. DNBS + BJe (10 mg/kg): Mice were subjected to DNBS administration described as above, and BJe (10 mg/kg) was administered by oral gavage every 24 h, for 4 days starting from 1 h after the administration of DNBS.
8. DNBS + BJe (20 mg/kg): Mice were subjected to DNBS administration described as above, and BJe (20 mg/kg) was administered by oral gavage every 24 h, for 4 days starting from 1 h after the administration of DNBS.

The doses of BJe were based on a previous study [17]. The dose (20 mg/kg) showed more beneficial effects compared to 5 and 10 mg/kg and was used for immunohistochemistry and western blot analysis.

2.5. Evaluation of colon damage

After its removal, the entire colon was gently rinsed with saline solution, opened by a longitudinal incision and immediately examined under a microscope. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers as described previously [18] according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5–8, one point is added for each centimeter of ulceration beyond an initial 2 cm.

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