Passiflora edulis leaf extract inhibits inflammatory response and preserves intestinal barrier function in Caco-2 and RAW264.7 co-culture model

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Abstract

Inflammatory bowel disease (IBD) is a chronic and incurable illness that affects people all over the world. Conventional therapies usually cause adverse side effects that may affect patients’ quality of life. This study assessed the effect of the aqueous extract of Passiflora edulis leaves (PELE) in co-culture of Caco-2 and RAW264.7 cells, simulating an in vitro model of IBD. After being stimulated with bacterial-derived lipopolysaccharides (LPS), PELE’s treatment inhibited the release of the pro-inflammatory cytokines interleukin (IL) 6 and 8, although it was not able to reduce nitric oxide (NO) production. In the co-culture system, PELE was also able to preserve the intestinal barrier function by decreasing paracellular permeability and increasing transepithelial electrical resistance (TEER) values, compromised after the LPS stimulus. The beneficial activity seen in this in vitro study may suggest that PELE has a possible anti-inflammatory role in IBD and can alleviate inflammatory events.

Keywords: gut inflammation; Passiflora edulis leaf extract; intestinal bowel disease; intestinal barrier.

Practical Applications: Passiflora edulis has been widely explored due to its health benefits. The benefits extend beyond the fruit; its leaf can also have positive effects on gut health. This study shows an anti-inflammatory role of PELE aqueous extract in inflamed intestinal and macrophages cells. This finding could contribute to the discovery of new adjuvant therapies for IBDs.

1 INTRODUCTION

Worldwide, more than 3.5 million people are affected by inflammatory bowel disease (IBD), and their occurrence has grown successively (Kaplan, 2015). The most common forms of IBD are Crohn's disease and ulcerative colitis, which are characterized by recurrent and remitting chronic inflammation of the entire gastrointestinal tract or only the colon, respectively (Lloyd-Price et al., 2019). The pathophysiology of IBD is not fully understood, however there are evidences that damaged intestinal barrier; dysbiosis; persistent infection; and inflammation could increase the immune response, permitting the translocation of luminal antigens into the bowel wall (Machado et al., 2021; Silva et al., 2016). Conventional therapies for IBD – usually based on corticosteroids, aminosalicylates, antibiotics, and immunomodulatory drugs – focus on inducing remission and involve long treatments, which can cause adverse side effects (Katsanos et al., 2019).

Polyphenols are commonly existent in fruits, vegetables, and some leaves (Albuquerque et al., 2019). Owing to its anti-inflammatory and antioxidant characteristics, they could be useful as IBD's therapy. Phenolic compounds can raise intestinal health mainly through intestinal barrier defense and modulation of the inflammation response (Li et al., 2020). Additionally, polyphenols are capable of triggering assorted antioxidant and protective genes via nuclear transcription factors (Garcia-Lafuente et al., 2009); and they may impair the initiation of the nuclear factor κB pathway and other inflammatory pathways, thus decreasing the release of pro-inflammatory factors (Liu et al., 2020). Besides, they could also augment the antioxidant ability and enhance the growth and adhesion of probiotics (Barbosa et al., 2020; Carmo et al., 2020a).

Passiflora edulis leaves (PELE) exhibit anti-inflammatory and antioxidant activities (Cazarin et al., 2015; Montanher et al., 2007; Silva et al., 2013), among other properties, which could be mostly related to the occurrence of polyphenols (Colomeu et al., 2014; He et al., 2020). In rats with induced colitis, the consumption of PELE extract reduced the level of pro-inflammatory cytokines in the colon, mainly interleukin (IL) 1β and tumor necrosis factor α (TNF-α) (Cazarin et al., 2015). In Caco-2 cells monolayers with inflammatory stimuli such as IL-1β and bacterial-derived lipopolysaccharides (LPS), PELE was able to reduce IL-8 production and recover barrier dysfunction, protecting intestinal epithelial integrity (Carmo et al., 2020b).

Caco-2 cells are established in vitro models to predict transport and permeability features similar to in vivo studies (Ding et al., 2021; Van De Walle et al., 2010). However, this model does not take into account the other types of cell existent in the...
intestine. Thus, in this study, we used a co-culture of intestinal epithelial cells (Caco-2 cells) and macrophages (RAW 264.7 cells) to mimic the gut in inflamed states, maximizing the applicability of this in vitro model. Moreover, this study examined the effect of PELE on the improvement of gut inflammation.

2 MATERIALS AND METHODS

2.1 Chemicals

Phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin – 10 mM EDTA solution and solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, and fetal bovine serum (FBS) were acquired from Gibco (Waltham, MA, USA). Lucifer Yellow (LY) was bought from Thermo Fisher Scientific (Waltham, MA, USA). Cell culture flasks, plates, and 12-well transwell inserts (1.13 cm² and 0.4 µm pore size) were purchased from Greiner Bio-One (Monroe, NC, USA). LPS and N-(1-naphthyl) ethylenediamine were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Sulfanilamide and phosphoric acid were purchased from Dinâmica (Indaiatuba, SP, Brazil). The enzyme-linked immunosorbent assay (ELISA) kit for IL-8 and IL-6 determination was purchased from BD OptEIA™. The water was Milli-Q ultrapure (Merck Millipore, Darmstadt, Germany).

2.2 Sample preparation and characterization

PELE were prepared as described earlier (Carmo et al., 2020b). Briefly, they were obtained from the Agronomic Institute of Campinas (Campinas, SP, Brazil), washed under running water, dehydrated in an air circulation oven (50 °C and 48 h), milled to a fine homogeneous powder, and stored at -80 °C until extraction. To prepare the extract, we made an infusion similar to a homemade tea. After the purification step was made and all analyses were made with non-concentrated aqueous extract, to try to see the possible pharmacological effects of these extracts in the simulation of a homemade tea.

The polyphenol profile of PELE was previously analyzed (Silva et al., 2013). The estimation of the antioxidant capacity and the assessment of total phenolic were also estimated and published (Carmo et al., 2020b).

2.3 Cell culture maintenance

Caco-2 cells were acquired from “Banco de culturas do Rio de Janeiro” and RAW267.4 cells were kindly donated by Dr. Wirla Maria da Silva Cunha Tamashiro, from the Institute of Biology of UNICAMP. Both cell types were cultivated in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS, called DMEM-10% from now on, in an atmosphere of 5% CO₂ and 90% relative humidity at 37 °C. Cells grew on 75 cm² flasks, and they were used between passages 20 and 40. The medium was replaced every 48 h and the cells were subcultured every week by treatment with trypsin-EDTA solution.

2.4 Determination of cell viability

For viability experiments, Caco-2 cells were plated at a density of 3 × 10⁴ cells/cm² in 96-well plates and incubated for 24 h at 37 °C. The cells were treated with different concentrations of the PELE diluted in DMEM (0.2–100 mg/mL) and incubated for 24 h. After this period, all media were removed, and 100 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, Missouri, USA) solution at 5 mg/mL in PBS (Life Technologies, Carlsbad, CA, USA) was added to the cell culture. The cells were further incubated at 37 °C for 1.5 h. The formazan crystals were dissolved by incubation for 30 min with dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Missouri, USA). The optical density of formazan solution was measured (540 nm) and the results were expressed as percentage of the control (cells without any treatment).

2.5 Inflammatory assay

2.5.1 Caco-2

Caco-2 cells were plated (1×10⁵ cells/cm²) on transwell inserts in DMEM-10% and allowed to grow for 14 days for complete differentiation. The transepithelial electrical resistance (TEER) was determined on Caco-2 monolayers employing a Millicell® ERS voltohmmeter (EMD Millipore, Eschborn, Germany). The measurement was made in quadruplicate. The experiment was started when the TEER values were higher than 400 Ω cm² (Leonard et al., 2010).

On the basolateral side, LPS (2 µg mL⁻¹ in DMEM with 1% FBS) was added as a pro-inflammatory factor for 24 h. Once LPS was eliminated, the cells were washed twice with PBS, and then DMEM containing 1% FBS (DMEM-1%), as a positive control (PC), or PELE were added (5 and 10 mg/mL in DMEM-1%) in the apical side for more 24 h. Thus, after incubation, the basolateral medium was collected for determination of inflammatory markers. The negative control (NC) was made with just DMEM-1% during the experiment, without inflammatory stimulus or samples. An extra PC received the anti-inflammatory medicine budesonide (BUDE, 1 µM), instead of PELE.

2.5.2 RAW264.7

RAW264.7 cells were plated in 24-well plates (5×10⁴ cells/cm²) in DMEM-1% for 24 h. Subsequently, DMEM with 1% LPS (2 µg mL⁻¹) was added for 48 h. After LPS' inducement, cells were washed twice with PBS, and then DMEM-1% (PC) or PELE treatment was applied (5 and 10 mg/mL in DMEM-1%) for 24 h. Afterward, the supernatant medium was collected for determination of inflammatory markers. The NC and the extra PC followed the same specifications as Caco-2 inflammatory assay.
2.5.3 Co-culture

A co-culture model established by Tanoue et al. (2008) was used. Briefly, Caco-2 cells were plated in the same conditions described above (Section 2.5.1). After 21 days, RAW264.7 cells (5x10⁴ cells/cm²) were seeded at the basolateral side, and they were incubated for an additional 24 h. Then, LPS (2 μg mL⁻¹ in DMEM-1%) was added in the basolateral side and the plate was incubated for 24 h. After LPS’ inflammatory inducement, the co-culture was washed twice with PBS, and then DMEM-1% (PC) or PELE samples were added to the apical side (at 5 and 10 mg mL⁻¹ in DMEM-1%). At the same time, LY (1 mg/mL) was added to the transwell on the apical side, to evaluate Caco-2 monolayer barrier. Finally, the co-culture was incubated for 24 h. The NC and the extra PC followed the same specifications as the Caco-2 inflammatory assay. At the final point of the experiment, the supernatants were collected and assayed for inflammatory markers. The fluorescence of basolateral was measured (ex: 430 nm/em: 540 nm), and it was used to determine the rate of LY on the basolateral side as apparent permeability (Papp) using the Equation 1:

\[ P_{app} = \frac{\Delta Q/\Delta t}{A C_0} \]  

Where:
\( \Delta Q/\Delta t \): the linear appearance flux of LY on the basolateral side;
A: the membrane surface area (cm²);
C₀: the initial concentration of LY in the apical compartment.

The integrity of the Caco-2 monolayer was assessed by the TEER measurement, using Millicell-ERS voltohmmeter (EMD Millipore, Eschborn, Germany), in quadruplicate.

2.5.4 Analysis of IL-6 and IL-8 secretion and nitric oxide (NO) levels

The supernatants were recovered and assayed for IL-8 and IL-6 liberation using ELISA kits, following the manufacturer’s instructions. The measurement was performed in quadruplicate. NO concentration was also determined in supernatant using Griess’ reagent (1% sulfanilamide, 5% phosphoric acid, and 0.1% N-(1-naphthyl) ethylenediamine), according to Griess reaction (Green et al., 1982).

2.6 Statistical analysis

The statistical difference among groups was evaluated using analysis of variance (ANOVA), followed by post-hoc Tukey’s honestly significant difference. Data are expressed as mean±standard deviation (SD) and P<0.05 was considered significant. The statistical analyses were performed using Graph Pad Prism 7.0 software (Graph-Pad Software, Inc.).

3 RESULTS

3.1 Effects of PELE on cells viability

The MTT trial was carried out to assess Caco-2 and RAW264.7 viability. We used 0.2–100 mg/mL of PELE and viable cells conditions were considered those where reductions in viability ≤20% was observed. In both strains, the loss of viability was observed from the concentration of 30 mg/mL. Therefore, the inflammatory assays performed using 5 and 10 mg/mL concentrations were considered safe.

3.2 Anti-inflammatory properties of PELE in RAW264.7 and in Caco-2 cells

The extracts at both concentrations were efficient in reducing the pro-inflammatory cytokine IL-6 (23.9% for 5 mg/mL and 17.2% for 10 mg/mL, in respect with the PC — Figure 2A). The treatment with BUDE presented the highest levels of IL-6 reduction (34.3%). Regarding IL-8, both PELE’s treatment and BUDE had similar reduction levels (55.8, 37.6, and 59.4%, respectively, Figure 2B). PELE treatment was not effective in reducing NO levels in RAW264.7 cells (Figure 2C).

The incubation with PELE inhibited the liberation of IL-6 in Caco-2 cells (10 mg/mL: 22.5% and 5 mg/mL: 34.7%, Figure 3A), plus 5 mg/mL treatment showed no statistical difference with BUDE (40.3%). Regarding the secretion of IL-8 (Figure 3B), PELE 5 mg/mL and BUDE promoted similar levels of reduction (63.3% and 70%, respectively). Notably, 10 mg/mL treatment also presented a significant reduction, although a little higher than
the other treatments (52%). For NO production, PELE did not show statistical difference to PC (Figure 3C).

### 3.3 Effects of PELE in co-culture

The treatment with PELE extracts was effective in decreasing the liberation of IL-6 to co-culture in all treatments (54.6%, 59%, and 72.1% for PELE 5, PELE 10, and BUDE, respectively; Figure 4A). The PELE extract also reduced IL-8 production (97.3 and 95.2%, for 5 and 10 mg/mL, respectively, Figure 4B), in levels similar to BUDE (95.5%). Regarding NO production, treatments with the PELE were not able to reduce it. Meanwhile, the treatment with BUDE attenuated NO production by 48.7%, similar to 10 mg/mL treatment's reduction (34.3%, Figure 4C).

Regarding the Caco-2 monolayer barrier, PELE extract was able to reduce the paracellular Papp in both concentrations. Notably, 10 mg/mL was more effective (67.4% reduction) than 5 mg/mL (31.7%) and similar to BUDE treatment (82.6%, Figure 5A). This effect can also be seen in TEER values (Figure 5B). LPS NC: negative control; PC: positive control (LPS, 2 μg/mL); PELE 5: LPS (2 μg/mL) and PELE (5 mg/mL); PELE 10: LPS (2 μg/mL) and PELE (10 mg/mL); BUDE: budesonide (1 μM).

**Figure 2.** Effect of PELE on the reduction of cytokines and NO concentration in RAW264.7 cells. (A) IL-6 secretion. (B) IL-8 secretion. (C) NO secretion. Data are expressed as mean±SD (n=4) and were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Different letters mean statistical difference (P<0.05).

**Figure 3.** Effect of PELE on the reduction of cytokines and NO concentration in Caco-2 monolayer. (A) Levels of IL-6. (B) Levels of IL-8. (C) Production of NO. Data are expressed as mean±SD (n=4) and were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Different letters mean statistical difference (P<0.05).

**Figure 4.** Effect of PELE on the reduction of cytokines and NO concentration in Caco-2/RAW264.7 co-culture. (A) Levels of IL-6. (B) Levels of IL-8. (C) Production of NO. Data are expressed as mean±SD (n=4) and were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Different letters mean statistical difference (P<0.05).
greater values than NC, showing a protective effect. and BUDE were able to increase TEER in similar or in similar or stimulus decreased TEER in 33.7%. PELE in both concentrations post-hoc test. Different letters mean statistical difference (P<0.05). (n=4) and were analyzed using one-way ANOVA followed by Tukey's Figure 5. PELE's effect on intestinal barrier in the Caco-2/ co-culture. (A) Papp, (B) TEER final point. Data are expressed as mean ± SD (SD (n=4) and were analyzed using one-way ANOVA followed by Tukey's (A) Papp, (B) TEER final point. Data are expressed as mean ± SD (n=4) and were analyzed using one-way ANOVA followed by Tukey's．

4 DISCUSSION

In this study, we assessed the anti-inflammatory in vitro response of PELE in the intestinal epithelium (Caco-2 cells), in macrophages (RAW264.7), and in a co-culture model with both strains. The co-culture was the most responsive model in terms of pro-inflammatory ILS's release. When incubated with LPS (PC), they released greater amounts of IL-6 and IL-8 (44 and 83 fold changes, in respect to NC, respectively), when compared to Caco-2 cells (IL-6: 18 and IL-8:16 fold changes) and to RAW264.7 (IL-6: 37 and IL-8: 14 fold changes) alone. Macrophages are the second most abundant kind of cell existing in the intestinal epithelium and are essential in the inflammatory cascade, encouraging the release of pro-inflammatory cytokines, as well as the generation of reactive oxygen species (León-Rodríguez et al., 2019). Although in vitro experiments did not entirely mimic in vivo physiology and complexity, the co-culture could be considered a better model to explore the role of bioactive compounds in IBD, as the macrophage cells used in the co-culture are stimulated by LPS to produce a pool of pro-inflammatory cytokines that will challenge the intestinal cells, simulating an inflamed bowel environment.

After the inflammatory stimulus, PELE were able to reduce IL-6 and IL-8 release in similar levels of the anti-inflammatory drug BUDE. Cytokines are being directly associated in the inflammatory response. The co-culture model was able to produce a great amount of NO when incubated with LPS (102 fold changes, in comparison to NC); however, PELE was not able to modulate NO secretion in none of the three in vitro models used. Although NO is increased in patients with IBD (Cross & Wilson, 2003), it has a controversial function in the intestinal inflammation (Mu et al., 2019). Inhibition of NO increased tissue damage or dysfunction in some models of inflammation, meanwhile NO was beneficial in others models of inflammation (Kubes & McCafferty, 2000). Plus, NO can have a protective protagonist against intestinal barrier dysfunction, by changing the glutathione (GSH)/GSSG equilibrium resulting in the inactivation of phosphatases and protein tyrosine phosphorylation (Mu et al., 2019).

Barrier dysfunction is a critical determinant in the inflammatory processes at the intestinal, and it is accepted as an early episode in the pathogenesis of IBD. Changes in intestinal permeability could increase the inflammatory cascade as the consequence of a discrepancy among pro- and anti-inflammatory cytokines released by immune cells (Neurath, 2014). Therefore, keeping the epithelial barrier function is a key for IBD’ treatments. PELE preserved Caco-2 monolayer integrity in the co-culture system, decreasing paracellular permeability and increasing TEER values, compromised after LPS stimulus. Other studies have also shown a protective role of polyphenols’ rich extracts in inhibiting the disruption of the intestinal barrier (Kim et al., 2021; Valdez et al., 2020).

PELE was previously analyzed, and its phenolic profile is already known (Silva et al., 2013). Briefly, the three more abundant flavonoids presented in PELE were vitexin, isovitexin, and isoorient (0.40, 0.50, and 1.05 mg/g, respectively). Some studies have pointed out the isoorient’s anti-inflammatory potential, reducing the expression IL-6, TNF-α, and IL-1β in vitro (Anilkumar et al., 2017; Wedler et al., 2014), regulating the release of TNF-α, IL-1, and IL-6 in overweight mice (Yuan et al., 2016), and attenuating colon-induced damage and gut microbiota dysbiosis in mice (He et al., 2019). Thus, PELE’s phenolic composition could partially explain its anti-inflammatory response.

The beneficial activity seen in this in vitro study may suggest that PELE has a possible anti-inflammatory role in IBD, and could minimize inflammatory events. However, this study presents some limitations that should be considered. There are several factors and cell types that will be presented in vivo that is not represented in the model used – it does not include others intestinal and/or immune cells either the intestinal microbiota or the digestive process it will go through, that could affect the bioavailability and metabolites production of the polyphenols presented in PELE. Furthermore, plausible inter-species cross-reactions must also be considered, as this model combines murine and human cell lines (León-Rodríguez et al., 2019). Therefore, the anti-inflammatory effect of PELE...
should be confirmed in immune cells from humans activated by inflammatory inducement and, further, must be validated in human objects.

5 CONCLUSION

PELE extract can weaken intestinal inflammation by reducing the pro-inflammatory cytokines IL-6 and IL-8, restoring the intestinal barrier that was compromised by the LPS stimulus in a co-culture in vitro model with Caco-2 and RAW264.7 cells that take into account the crosstalk between immune cells and intestinal epithelial cells.

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