PRECLINICAL EVIDENCE FOR THE THERAPEUTIC POTENTIAL OF *Physalis peruviana* L TO TREAT INFLAMMATORY BOWEL DISEASE.

Doctoral thesis by

Yanet Cecilia Ocampo Buendía

Doctoral program in Biomedical Sciences

Institute for Immunological Research

University of Cartagena

Universidad de Cartagena
Fundada en 1827

PRECLINICAL EVIDENCE FOR THE THERAPEUTIC POTENTIAL OF *Physalis peruviana* L TO TREAT INFLAMMATORY BOWEL DISEASE.

Doctoral Thesis by
Yanet Cecilia Ocampo Buendía

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

Supervisor
Luis Alberto Franco Ospina, Ph.D.

University of Cartagena
Institute for Immunological Research
Ph.D. Program in Biomedical Sciences

Cartagena, Colombia
2017

To my Lord and Savior: Jesus Christ

"And whatever you do in word or deed, do all in the name of the Lord Jesus, giving thanks to God the Father through Him". Colossians 3:17-21

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

ABBREVIATIONS

- IBD: Inflammatory bowel disease
- TNBS: Trinitrobenzenesulfonic acid
- MPO: Myeloperoxidase
- IL: Interleukin
- TNF: Tumor necrosis factor
- NO: Nitric Oxide
- iNOS: Inducible nitric oxide synthase
- COX: cyclooxygenase
- NF-κB: Nuclear factor κB
- Muc: Mucin
- CD: Crohn's disease
- UC: Ulcerative colitis
- Th: T helper
- IFN: Interferon
- DSS: Dextran sodium sulfate
- TLR: Toll like receptors
- NOD: Nucleotide oligomerization domain
- STAT: signal transducer and activator of transcription
- MAPKs: mitogen-activated protein kinases
- PPARγ : Peroxisome proliferator-activated receptor gamma
- LRR: Leucine-rich repeat
- MDP: Muramyl dipeptide
- ATG16L1: Autophagy-related protein 16-1
- UPR: Unfolded protein response
- ER: Endoplasmic reticulum
- GWAS: Genome wide associated studies
- XBP1: X-box-binding protein 1
- Treg: Regulatory T cells
- TGF: Transforming growth factor
- JAK2: Janus kinase 2
- FDA: Food and drugs administration
- LPS: Lipopolysaccharide
- PGE2: Prostaglandin E2
- TPA: 12-O-tetradecanoyl-phorbol-13-acetate
- PDA: Photodiode array
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

- LD_{50}: Lethal Dose 50
- IC_{50}: Inhibitory concentration 50
- HPLC: High performance liquid chromatography
- NMR: Nuclear magnetic resonance
- MS: Mass spectrometry
- DMEM: Dubelco's modified eagle medium
- DPPH: 2,2-diphenyl-1-picrylhydrazyl
- ABTS: 2,2'-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid
- Trolox: 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid
- PVP: Polyvinylpyrrolidone
- FBS: Fetal bovine serum
- PAS: Periodic Acid-Schiff
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- PBS: Phosphate buffer saline
- H&E: Hematoxylin and eosin
- MMC: Mitomycin C
- SG: Specific gravity
- ALT: Alanine transaminase
- AST: Aspartate transaminase
- ALP: Alkaline phosphatase
- PCE: Polychromatic erythrocytes
- NCE: Normochromatic erythrocytes
- MN: Micronuclei
- OTM: Olive tail moment

ABSTRACT

Phytotherapy constitutes an emerging alternative strategy for the treatment of inflammatory bowel disease (IBD) that combine efficacy and an adequate safety profile. Although medicinal plants are integral part of Colombian culture, only few of them have been studied deeply. One example is *Physalis peruviana* (Cape gooseberry), which has previously demonstrated anti-inflammatory activity. This doctoral thesis aimed to investigate the therapeutic potential of *P. peruviana* calyces to treat IBD, using trinitrobenzenesulfonic acid (TNBS)-induced rat colitis, a well characterized experimental model with some resemblance to human IBD. In order to do so, the study started with the isolation and identification of the main anti-inflammatory compounds of the total ethereal extract using column chromatography and spectroscopy, and continued with the evaluation of the effect of total extract and pure compounds on TNBS-induced colitis after 3 days (preventive set-up, acute model) and 15 days (therapeutic set-up, chronic model) of colitis induction. At the end of each experiment, colonic inflammation was evaluated measuring macroscopic/histologic damage, MPO activity, changes in cytokines levels, and gene or protein expression. In addition, the safety of long-term administration of isolated compounds was evaluated using a toxicity study in mice, including necropsy, histology, hematology, serum biochemistry, gene expression, micronucleated erythrocytes, and DNA damage, as toxicological endpoints. The chemical study of the total extract of *P. peruviana* led to the purification of a mixture of two new sucrose esters: Peruviose A and B, which only differ in the substituent at the C3 position of fructose: isobutyryl and 3-methyl-butanoyl, respectively. Pharmacological studies demonstrated the intestinal anti-inflammatory activity of *P. peruviana* total extract with a significant improvement in the colonic tissue at both macroscopic and histological levels, along with a significant reduction of IL-1β and TNF-α. Similarly, peruviose A and B, when administered for two weeks, remarkable ameliorated TNBS-induced colitis, promoting the inhibition of inflammatory mediators (MPO, NO, TNF-α, IL-1β, IL-6, IL-10, IL-17), gene (iNOS, COX-2, NF-κB), and protein (nuclear NF-κB and iNOS) expression, while increasing MUC-2 mRNA expression. Although the 3 days treatment with total extract or sucrose esters from *P. peruviana* did not produced a potent anti-inflammatory effect, it was sufficient to significantly reduce the extent and severity of tissue damage as well as microscopic disturbances. Taken together, these results provide the first evidence of intestinal anti-inflammatory activity of *P. peruviana* calyces positioning sucrose esters as the main bioactive compounds to effectively treat IBD. With regard to toxicological evaluation, the results showed no significant differences between treated animals and control group in any evaluated parameter. Thus, peruvioses A and B can be safely employed at therapeutic dosage levels. Overall, this doctoral thesis provides encouraging and sufficient evidence to start a clinical study to demonstrate the efficacy of *P. peruviana* as individual or adjuvant treatment for IBD.

1 CHAPTER ONE. Thesis Overview

Inflammatory Bowel Disease (IBD) represents a group of chronic, relapsing, and remitting inflammatory disorders of the gastrointestinal tract, that affect millions of people worldwide.[1] Because of the lack of efficacy and poor tolerability of conventional drugs, the employment of complementary and alternative medicines, especially herbal drugs for the management of IBD is increasing.[2] In this chapter, the conception of the thesis project, including the hypothesis and objectives are presented. Since this research is based on the rationale for *P. peruviana* to have a positive impact on intestinal inflammation, chapter 2 will discuss the pathogenesis and treatments available for IBD, together with a brief description of botanical aspects and ethnopharmacological employment of *P. peruviana*. The chemical, pharmacological, and toxicological study of *P. peruviana* was developed in several stages and results are described in separate chapters (3-6). Such chapters are the center of attention in this document and were prepared using the general format acceptable for most scientific publications (abstract, introduction, methods, results, discussion, and conclusion). In chapter 3, the isolation, purification, and structural elucidation of the major bioactive compounds of *P. peruviana* calices is described, as well as the evaluation of their acute toxicity and biological effect in a model of general inflammation (λ-carrageenan paw edema). Subsequently, the beneficial effect of total extract (chapter 4) and sucrose esters (chapter 5) from *P. peruviana* in the rat model of colitis induced by (TNBS), and the molecular targets involved in their bioactivity are discussed. Results in chapter 6 demonstrate the safety of sucrose esters from *P. peruviana* in a 28-day repeated-dose study in mice. Finally, a general discussion about the main contributions of this thesis is presented in chapter 7, in addition to a section for concluding remarks and future perspectives. The Appendix includes a list of publications related to this doctoral thesis; a copy of the IR, FAB-MS, and NMR spectra used for structural elucidation; proof of the approval by the Ethics Committee; as well as 2 tables describing the histological analysis and primers used to evaluate gene expression.

1.1 Study Conception and Pertinence

IBD is classified in two major forms, Crohn's Disease (CD) and Ulcerative Colitis (UC), which are distinguished by their clinical, histological, immunological and genetic features.[3, 4] CD develops mostly in the terminal ileum and colon but can affect different portions of the gastro-intestinal tract; the lesions are patchy and segmental, and inflammation typically transmural with formation of deep fissuring ulcers, non-caseating granulomas, fistulae or intestinal strictures.[5, 6] Inflammation is oriented to a T helper (Th)1 and Th17 immune response, characterized by an enhanced expression of interleukin (IL)-12/IL-23 and interferon (IFN)γ/IL-17 [7, 8]. In contrast, key features of UC include a purely mucosal and continuous inflammation restricted to colon and rectum. Histologically UC is characterized by depletion of goblet cells and the presence of a significant number of

neutrophils within the lamina propria and the crypts, where they form crypt abscesses.[6] Unlike CD, inflammation is oriented to a Th2 response with excess of IL-5 and IL-13 production.[8, 9] However, regardless of these differences both UC and CD patients have increased levels of tumor necrosis factor (TNF)-α and several pro-inflammatory cytokines involved in innate and adaptive immune responses such as IFN-γ, IL-1β, IL-6, IL-12 and IL-17.[6, 7] Cytokines have been directly implicated in the pathogenesis of IBD in recent genetic and immunological studies, and they seem to have a crucial role in controlling intestinal inflammation and the associated clinical symptoms of IBD.[10]

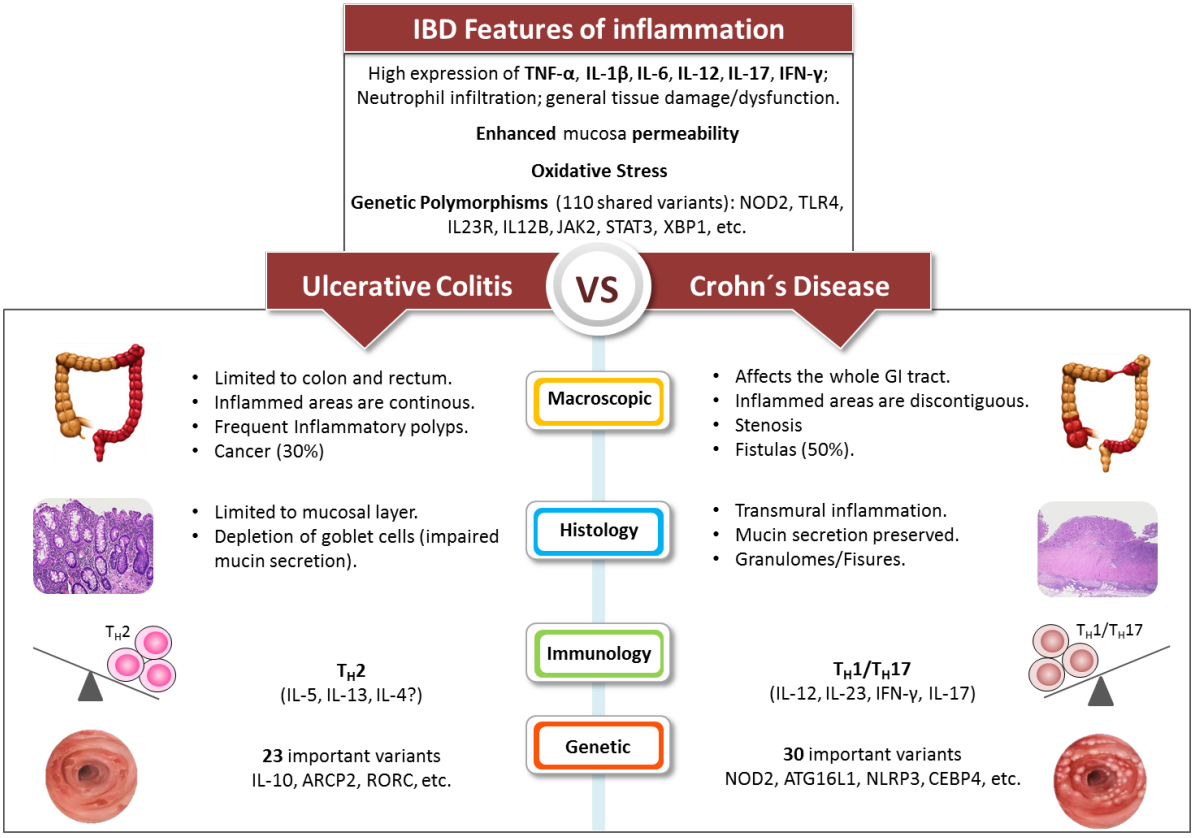


Figure 1.1. Main pathophysiological features of IBD. In the upper part, general features of IBD are presented; cytokine/chemokine unbalance, leaky gut, and oxidative stress are important targets for pharmacological intervention. The lower part summarizes specific features of ulcerative colitis (UC) and Crohn's Disease (CD). Although UC and CD are defined clinicopathological subtypes, specific immunological differences are still debatable. Abbreviations; ARPC2, actin related protein 2/3 complex, subunit 2; ATG16L1, autophagy related 16-like 1; CEBP4, Cytoplasmic polyadenylation element binding protein 4; IL-12B, IL-12 beta; IL-23R, IL-23 receptor; JAK2, Janus kinase 2; NLRP3, NLR family pyrin domain containing 3; NOD2, Nucleotide-binding oligomerization domain-containing protein 2; RORC, RAR related orphan receptor C; STAT3, signal transducer and activator of transcription 3; TLR4, toll like receptor 4; XBP1, X-box binding protein 1. Adapted from [8]

Both disorders are associated with marked morbidity and no medical cure.[11] Current pharmacological strategies to reduce the chronic inflammation in the intestinal mucosa

include aminosalicylates, corticosteroids, thiopurines, methotrexate, cyclosporine, and biologics such as anti-TNFα.[12] A huge drawback is that long-term employment of these agents produce systemic immunosuppression with serious clinical complications, [13] which highlights the need for optimized anti-inflammatory therapy.

In this context, the employment of alternative therapies, including herbal medicines, is increasing and gaining popularity worldwide [14], since their effectiveness and safety are presumed. In parallel, the raised interest in the scientific community to evaluate alternative IBD drugs has led to studies showing natural products with the ability to neutralize pro-inflammatory cytokines/pathways to prevent and treat intestinal inflammation at both pre-clinical and clinical levels.[15-17] As a consequence, the investigation of herbal extracts and secondary metabolites from plants used in folk medicine constitutes an invaluable strategy to develop new therapies for IBD. Nevertheless, the success in this field has been significantly limited by questionable research with poor chemical characterization of active compounds, insufficient pre-clinical studies using animal models, unclear mechanism of action, small size of tested patients and short time of evaluation.[12]

The laboratory of Biological Evaluation of Promising Substances recently started a program to study anti-inflammatory extracts and compounds from Colombian medicinal plants. As a result, we identified *Physalis peruviana* L. calyces as an important source of immunomodulatory compounds.[18] Indeed, this species is widely used in South American folk medicine to treat malaria, asthma, hepatitis, dermatitis, and rheumatoid arthritis.[19] Considering this background, this doctoral thesis was conceived to investigate the effect of *P. peruviana* calyces during intestinal inflammation trough the employment of animal models and *in vitro* studies. This knowledge will add value to *P. peruviana* crops and will give a function to calyces which are currently treated as a waste product.

1.2 Hypothesis

Extract and compounds from the calyces of *P. peruviana* L. have immunomodulatory effects at intestinal level, and might constitute an effective and safe new strategy for the treatment of IBD.

1.3 Objectives

The main objective of this doctoral thesis was to study *P. peruviana* calyces to provide sufficient evidence of their efficacy to treat experimental IBD. Thus, our study was designed to accomplish the following specific objectives:

- To isolate, purify, and identify the major anti-inflammatory compounds from *P. peruviana* calyces using chromatography and spectrometry.

- To investigate the effect and protective mechanisms of the total extract and sucrose esters from *P. peruviana* calyces on TNBS-induced colitis in rats, at macroscopic, histologic, and biochemical level.
- To assess the toxicological effect of sucrose esters from *P. peruviana* following protocols for single and repeated dose administration.

1.4 References

1. Heylen, M., et al., *Of worms, mice and man: An overview of experimental and clinical helminth-based therapy for inflammatory bowel disease.* Pharmacology & Therapeutics, 2014. **143**(2): p. 153-167.
2. Rahimi, R., S. Nikfar, and M. Abdollahi, *Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: a meta-analysis.* World J Gastroenterol, 2013. **19**(34): p. 5738-49.
3. Xavier, R.J. and D.K. Podolsky, *Unravelling the pathogenesis of inflammatory bowel disease.* Nature, 2007. **448**(7152): p. 427-434.
4. Louis, E., C. Van Kemseke, and C. Reenaers, *Necessity of phenotypic classification of inflammatory bowel disease.* Best Pract Res Clin Gastroenterol, 2011. **25 Suppl 1**: p. S2-7.
5. Lichtenstein, G.R., S.B. Hanauer, and W.J. Sandborn, *Management of Crohn's disease in adults.* Am J Gastroenterol, 2009. **104**(2): p. 465-83; quiz 464, 484.
6. Monteleone, G., R. Caruso, and F. Pallone, *Targets for new immunomodulation strategies in inflammatory bowel disease.* Autoimmunity Reviews, 2014. **13**(1): p. 11-14.
7. Hisamatsu, T., et al., *Immune aspects of the pathogenesis of inflammatory bowel disease.* Pharmacology & Therapeutics, 2013. **137**(3): p. 283-297.
8. Dothel, G., et al., *Animal models of chemically induced intestinal inflammation: predictivity and ethical issues.* Pharmacol Ther, 2013. **139**(1): p. 71-86.
9. Cho, J.H., *The genetics and immunopathogenesis of inflammatory bowel disease.* Nat Rev Immunol, 2008. **8**(6): p. 458-66.
10. Neurath, M.F., *Cytokines in inflammatory bowel disease.* Nat Rev Immunol, 2014. **14**(5): p. 329-342.
11. Cosnes, J., et al., *Epidemiology and natural history of inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1785-94.
12. Li, R., et al., *An old herbal medicine with a potentially new therapeutic application in inflammatory bowel disease.* Int J Clin Exp Med, 2011. **4**(4): p. 309-19.
13. Jackson, L.N., et al., *Alternative medicine products as a novel treatment strategy for inflammatory bowel disease.* Am J Chin Med, 2008. **36**(5): p. 953-65.
14. Singh, U.P., et al., *Alternative medicines as emerging therapies for inflammatory bowel diseases.* Int Rev Immunol, 2012. **31**(1): p. 66-84.
15. Rahimi, R., S. Mozaffari, and M. Abdollahi, *On the use of herbal medicines in management of inflammatory bowel diseases: a systematic review of animal and human studies.* Dig Dis Sci, 2009. **54**(3): p. 471-80.
16. Hur, S.J., et al., *Review of natural products actions on cytokines in inflammatory bowel disease.* Nutr Res, 2012. **32**(11): p. 801-16.
17. Debnath, T., D.H. Kim, and B.O. Lim, *Natural products as a source of anti-inflammatory agents associated with inflammatory bowel disease.* Molecules, 2013. **18**(6): p. 7253-70.
18. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica, 2007. **27**(1): p. 110-5.
19. Lim, T.K., *Physalis peruviana*, in *Edible Medicinal And Non-Medicinal Plants*2013, Springer Netherlands. p. 300-309.

2 CHAPTER TWO. General Introduction

2.1 Inflammatory Bowel Disease (IBD)

2.1.1 Definition and Epidemiology

IBD is a heterogeneous group of illnesses, including ulcerative colitis (UC) and Crohn's Disease (CD), characterized by chronic and relapsing inflammation of the gastro-intestinal tract, which is mediated by a dysfunctional interaction of the tightly regulated crosstalk between lumen microbiota and mucosal immune system.[1, 2] The complex nature of IBD pathogenesis involves the interaction of genetic, environmental, microbial and immunologic factors that could intersect in several ways, altering intestinal homeostasis.[3, 4] Despite of this currently well-established view, our understanding of the disease is still limited, which is reflected in the uncertainty to what extent each factor contributes to the disease progression, and whether some are more important than others.[5, 6] Taking into account the objectives of this doctoral thesis, only a brief discussion of immunobiology of IBD will be presented in the next section of this chapter.

The incidence of IBD is dramatically increasing worldwide both in adults and pediatric patients.[7] The burden of this disease varies in different countries and populations, showing traditionally high-incidence in northern Europe, United Kingdom and North America.[8, 9] However, recent trends indicate a change in the traditional patterns of IBD epidemiology, with previously low-risk areas now reporting a continuous rise in incidence, including regions such as Asia, South America and southern and eastern Europe [10-12]; suggesting that changes in lifestyle, diet, urbanization and other environmental factors play an important etiological role in IBD. Indeed, disease incidence appears to increase in parallel to per capita income.[13]

Despite data from developing countries are generally scarce and lack methodological quality, most suggest low incidence rates. In the case of Colombia, there is not consistent data showing the current epidemiological situation of the country. Nonetheless, some studies suggest a similar trend with increasing incidence of IBD.[14, 15]

2.1.2 Immunobiology of IBD

In health, the intestinal immune system is tightly controlled, while in patients suffering from IBD, immune regulation is disturbed resulting in elevated expression of pro-inflammatory cytokines.[16] Available evidence suggests that dysregulated innate and adaptive immune pathways play a pivotal role in disease onset and maintenance.[17] In this section we attempted to describe the major perturbations that produce IBD, at both levels.

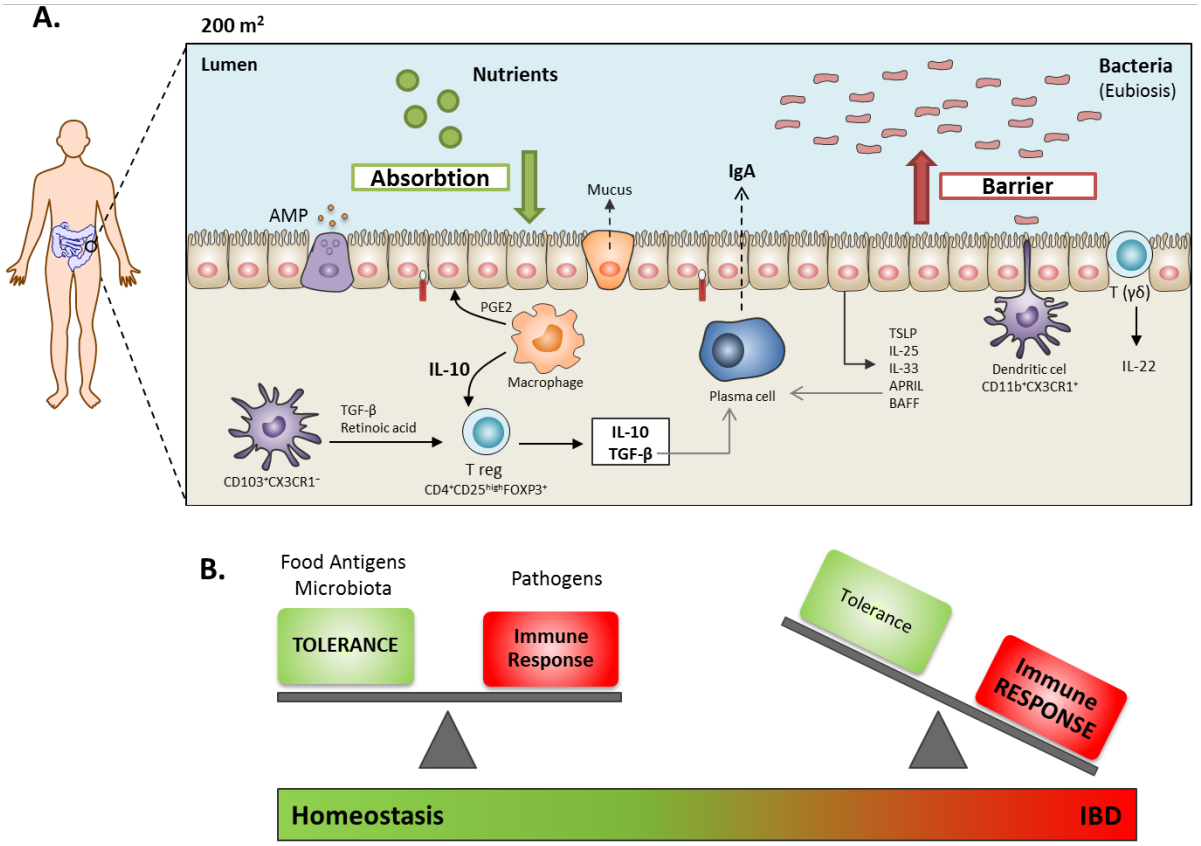


Figure 2.1. Intestinal Immune Homeostasis and Inflammation. From the immunological perspective, the intestinal mucosa has two remarkable features. First it has an extensive surface area (approx. 200 m^2) that allows maximum absorption of nutrients, water and electrolytes. Second the gut lumen is challenged with perpetual presence of commensal microbes. (A) In the presence of a balanced microbiota (eubiosis), the intestinal immune system induce and maintains tolerance to food microbes and food antigens through a complex set of innate and adaptive recognition strategies and effector mechanisms. Intestinal epithelial cells are essential for immune response with secretion of mucin (produced by goblet cells), antimicrobial peptides (AMPs – produced by Paneth Cells), epithelial cytokines (TSLP, IL-33, IL-25, BAFF, and APRIL) that promote development of tolerogenic dendritic cells (DCs; $CD103^+CX3CR1^-$) and macrophages (Mφ). Tolerogenic DCs and Mφ, in turn, induce the development of induced regulatory T cells (iTreg) through processes dependent on TGF-β and retinoic acid, or IL-10. Additionally, TGF-β from Tregs, as well as epithelial-derived BAFF and APRIL, promote generation of IgA plasma cells. Immune system also participates in the maintenance of epithelium integrity with secretion of PGE2 (Mφ and IEC) and IL-22 (γδ intraepithelial lymphocyte) (B) The equilibrium between tolerance Vs. Immune response is complex and fragile. In the case of IBD, alterations of the microbiota (dysbiosis), injury or xenobiotics, stimulate the production of pro-inflammatory cytokines that promotes intestinal inflammation and lack of resolution in genetically predisposed individuals. Abbreviations; APRIL, a proliferation-inducing ligand; BAFF, B-cell-activating factor of the TNF family; IBD, Inflammatory Bowel Disease; IgA, immunoglobulin A, PGE2, Prostaglandin E2; TGF- β, transforming growth factor; TSLP, thymic stromal lymphopoietin. Adapted from [18].

2.1.2.1 Innate immunity in IBD

The first physical barrier that intestinal bacteria and food antigens encounter on the mucosal surface is represented by the intestinal epithelium covered by a mucous layer.[19] An important characteristic shared by IBD patients is a dysfunctional intestinal epithelial barrier, which in turn facilitates translocation of harmful substances and pathogens to the lamina propria, promoting inflammation.[20] Alterations in the intestinal epithelium also promotes aberrant secretion of mucin compromising the thickness and activity of mucus layer.[21] In fact, experiments using $MUC2^{-/-}$ mice demonstrated an increased sensitivity to dextran sodium sulfate (DSS) when compared to wild type animals.[22]

Similarly, the expression of Toll Like Receptors (TLR), especially TLR4, is significantly increased in epithelial cells and lamina propria mononuclear cells (LPMNCs) during active IBD.[23, 24] Indeed, mutations of TLR4 are strongly associated to IBD development, particularly if they coexist with mutations of the nucleotide oligomerization domain 2 (NOD2) gene. Activation of TLR4 promotes the expression of nuclear transcription factor-kappa B (NF-κB), signal transducer and activator of transcription 1 (STAT1), mitogen-activated protein kinases (MAPKs), or Peroxisome proliferator-activated receptor gamma (PPARγ), promoting pro- as well as anti- inflammatory effects. Studies in mice support the hypothesis that mutations over-activating the TLR4 signaling promote intestinal inflammation via excessive production of cytokines (TNF, IL-1β, and IL-6).[25] On the other hand, mutations leading to functional loss of TLR4, or its downstream signaling, worsen DSS-induced colitis by disturbing intestinal homeostasis.[26, 27] Thus, dysfunction of TLR4 in both directions aggravates intestinal inflammation.[28]

NOD2 was the first susceptibility gene identified for CD and represents the best investigated and most well-established susceptibility gene for this disease.[29] Mutations within the NOD2 gene have only been associated with CD, but not UC, and are present in about 40% of CD patients.[28] These mutations are located in the C-terminal leucine-rich repeat domain (LRR), which is responsible for muramyl dipeptide (MDP) recognition.[30] In addition to the common NOD2 variants associated with CD [SNP8 (R702W), SNP12 (G908R) and SNP13 (1007fs)], other infrequent variables that are also localized to LRR have been discovered.[31]

Although the specific mechanisms by which disease-associated NOD2 polymorphisms contribute to the increased susceptibility to develop CD remains incompletely understood; some models have been proposed. First, it has been suggested that NOD2 negatively regulates TIR signaling, and hence NOD2 deficiency would promote excessive response to TLR ligands. Alternatively, the frame-shift variant (SNP 13) has been suggested to actively suppress IL10 transcription via inhibiting the nuclear ribonucleoprotein hnRNP-A1.[31] Finally, a third model proposes that mutated isoforms of NOD2 impair the production of α-defensins by Paneth cells, promoting alterations in gut microbiota composition.[30] This is

supported by Wehkamp *et al*., which reported a remarkable reduced expression of α-defensins in patients with ileal CD featuring NOD2 mutations (SNP13), when compared to control patients.[32]

Interestingly, recent evidence indicates that NOD2 is directly involved with autophagy trough physical interaction with autophagy-related protein 16-1 (ATG16L1), another CD susceptibility gene associated with Paneth cell dysfunction.[33] Indeed, gene variants of NOD2 and ATG16L1 are defecting in implementing proper autophagy.[34] Particularly, CD patients with T300A polymorphism of ATG16L1 display a large proportion of Paneth cells with abnormal granules; these abnormalities resemble those in mice with deficient expression of this protein.[35]

Another homeostatic cellular pathway essential for secretory cells, including Paneth cells and goblet cells, is the Unfolded Protein Response (UPR) that is triggered by Endoplasmic Reticulum (ER) stress to control proper folding of synthetized proteins.[29] UPR response might be mediated through IRE1/XBP1, ATF6p90/ATF6p50 and PERK/ATF4 pathways. In any case, this response reduces the rate of protein synthesis, decreases the load of proteins entering the ER, and increases the capacity of ER to handle unfolded proteins.[36] Recent genome wide associated studies (GWAS) showed a significant association of IBD with X-box-binding protein 1 (XBP1) polymorphisms. Kaser *et al* demonstrated that specific deletion of XBP1 in intestinal epithelial cells caused severe depletion of Paneth cells and goblet cells, whereas enterocytes remained completely intact.[37] XBP1 malfunction not only promotes dysbiosis by inhibiting the production of antimicrobial peptides, it also restrain the immune response to pathogens. As shown by Martinton *et al*, XBP1 is fundamental for the optimal response of TLR4 to its ligands, since UPR response allows the survival of inflammatory cells to the stress promoted by immune activation and cytokine production. Thus, XBP1 deficient mice developed spontaneous intestinal inflammation with ulceration, neutrophils infiltration, and crypt abscesses- main features of human IBD.[38]

2.1.2.2 Adaptive immunity in IBD

Although innate immune responses seem to be a prerequisite for the excessive activation of adaptive immunity, the latter undoubtedly drives the major tissue damage that is displayed in IBD patients.[39] Based mainly on the levels of T cell-derived cytokines detected in IBD mucosa, several studies have associated active disease with low proportion of regulatory T-cells (Treg) and abnormal development of activated T cells. It is classically considered that naïve CD4+ T cells are differentiated to T helper cell type (Th)1 in CD whereas UC is mediated by Th2 immune responses.[40] However, the notion that UC is a Th2-mediated disease remains controversial. For instance, UC patients produce low levels of IFN-γ instead of high expression of IL-4. Similarly, lower levels of IL-13 were found in the colonic mucosa of UC patients compared to CD patients and control subjects.[41]

CD Th1 cells, triggered by increased mucosal level of IL-12, produce high amounts of IL-2, IL-18, IFN-γ, and TNF-α, which in turn enhances the activation of intestinal macrophages.[17] Although CD has historically been considered a Th1 mediated disease, recent discovery of Th17 pathway has prompted to reconsider this paradigm, as key molecules associated with the development, function, and maintenance of Th17 cells are up-regulated in CD patients compared to healthy subjects.[40]

The pivotal role of Th17 cells in intestinal inflammation is not exclusive to CD. Indeed, recent data from GWAS have revealed several Th17 risk genes for IBD. The meta-analysis published by Jostins *et al.* demonstrated that variants of CCR6, STAT3, JAK2, IL-23R, and IL12B are associated to both UC and CD phenotypes and hence are included in the IBD genome.[42, 43] Th17 cells are characterized by the production of large amounts of IL-17A, IL-17F, IL-21 and IL-22, induced by IL-6 and transforming growth factor (TGF)-β, with expansion promoted by IL-23.[43] Following engagement of IL‑23R by IL‑23, Janus kinase 2 (JAK2) is activated, resulting in JAK2 autophosphorylation and phosphorylation of IL‑23R. This in turn results in the recruitment, phosphorylation, homodimerization and nuclear translocation of STAT3.[44] The variant R381Q of IL-23R has shown a high level of association to IBD susceptibility, probably by affecting JAK2 activity. Moreover, JAK2-STAT3 signaling is also related to IL-10 signaling, that is also involved in IBD pathogenesis.[45] An additional important gene is CCR6, expressed by various immune cells, mainly Th17 cells, promoting gut homing.[46] Overall, these evidence strongly implicate the IL-23 pathway and Th17 cells as central players in intestinal inflammation.

Intestinal homeostasis relays in Treg ($CD4^{+}CD25^{high}FOXP3^{+}$) and their ability to suppress abnormal immune response in the gut environment filled of microbiota and dietary antigens.[47] Interestingly, Treg cells are depleted in peripheral blood of patients with active IBD when compared to quiescent IBD and control subjects. Additionally, Treg exert a potent anti-inflammatory action in experimental colitis.[48]
Although, several mechanisms are involved in Treg immune suppression, the production of anti-inflammatory cytokines (IL-10 and TGF-β) is probably the most notorious.[47] Studies in mice highlight the importance of Il-10 and TGF-β expression during intestinal inflammation. However, L-10 has particular significance in IBD because IL-10 and IL-10R2 deficient mice do not develop lethal systemic autoimmunity, as seen in TGF-β1 deficient mice, but instead develop colitis. Moreover, established intestinal inflammation in mice can be ameliorated via treatment with recombinant IL-10 protein, IL-10 expressing transgenic T cells, or intestinal bacteria engineered to produce IL-10.[49]

Nevertheless, Treg implication in human IBD remains controversial since Treg are increased in the intestinal mucosa of IBD patients, and their function is normal, as demonstrated by the ability to suppress the proliferation of effector T cells. Recent studies suggest that the intestinal milieu in IBD patients alters Treg functional properties rendering them to a non-suppressive or even pathogenic phenotype. These hypothesis is supported from the observation that elevated production of IL-1, IL-6 and IL-23 in IBD mucosa, together with TGF-β, promotes the differentiation T cells into effector cells that sustain chronic inflammation.[47]

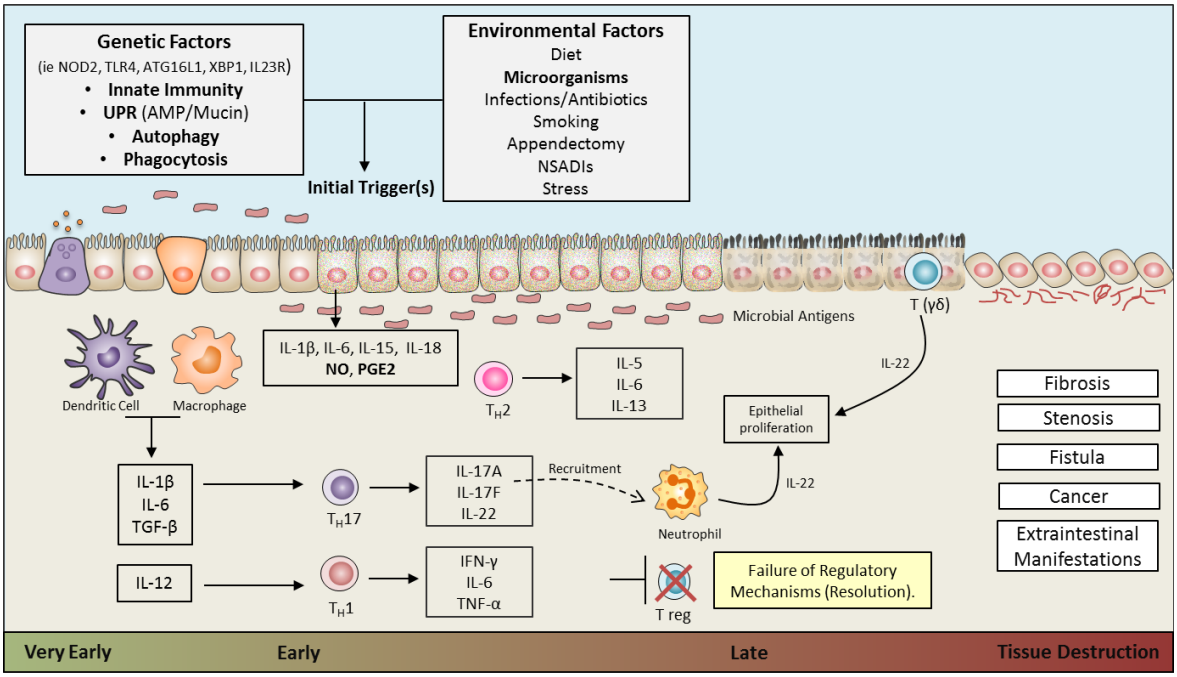


Figure 2.2. Pathogenesis of Inflammatory Bowel Disease. Although the exact etiology of IBD remains elusive, it is well recognized that genetic and environmental factors induce impaired barrier function, along with inadequate immune response. Initiating triggers that cause structural changes to the intestinal epithelium subsequently induces the translocation of bacteria and microbial products from the lumen to the lamina propria, which leads to innate immune cells activation in particular dendritic cells and macrophages, amplification of adaptive response by Th1, Th2, or Th17, and elevated pro-inflammatory molecules (NO and PGE2), cytokines/chemokines (IL-1β, IL-5, IL-6, IL-12, IL-13, IL-15, IL-17A/F, IL-18, IL-22, IFN-γ), and growth factors (TGF-β). If acute inflammation cannot be resolved by anti-inflammatory mechanisms, then chronic inflammation is developed with promotion of tissue destruction and causing complications such as fibrosis, stenosis, fistulas, cancer, and/or extraintestinal manifestations, which are common to all types of IBD. Abbreviations; AMP, antimicrobial peptides; IBD, inflammatory bowel disease; NSAIDs, non-steroidal anti-inflammatory drugs; NO, nitric oxide; PGE2, prostaglandin E2; mucosal effector T cells (T helper 1 (Th1), Th2 and Th17); UPR, unfolded protein response. Adapted from [16]

2.1.3 IBD Treatment

The primary goal of medical therapy for IBD patients is to suppress intestinal inflammation to provide relief of symptoms and achieve mucosal healing.[50] Routinely, treatment is

dependent on disease severity, localization, and associated complications. Regardless of this, medical management relays in pharmacological therapy and/or surgery to induce and maintain remission, while preventing complications (megacolon, abscesses, and fistula).[51]

Standard pharmacological therapy includes the use of aminosalicylates (5-aminosalicylic acid), steroids (prednisone and prednisolone), and immunosuppressants (cyclosporine, tacrolimus, azathioprine, and methotrexate).[51] Notwithstanding the cost of the treatment, these drugs do not induce complete clinical remission while producing serious secondary effects.[52] Under these circumstances, biological drugs appeared as a hopeful strategy for IBD management. Infliximab, a chimeric anti-TNF antibody, was the first biologic agent approved by the Food and Drugs Administration (FDA) in August of 1998. Subsequently, Adalimumab and Certolizumab pegol were approved. Since then, biological therapy has been increasingly employed in the treatment of IBD, showing a significant improvement for patient's clinical condition.[53] However, the evidence indicates that treatment with these molecules has not reduced the need of emergency surgery for patients with severe disease; instead it involves several risks and side effects. Renal complications, delayed hypersensitivity-like reaction, new onset of autoimmunity, and opportunistic infections are some examples of complications resulting from the immunosuppression induced by biological therapy.[52, 53] All these evidence, combined with the significant cost of biologic therapy compared to traditional drugs (236.370 Vs. 147.763 USD/patient), indicates the need for new therapies.[54]

Given the low efficacy and safety of standard therapies, the use of herbal medicines is rising globally. IBD patients favor the use of phytotherapy for two fundamental reasons: the desire of ending steroid consumption and the lack of confidence in their physician due to the unsatisfactory treatment.[55] In spite of the great potential of traditional herbal medicines, few high-quality investigations have been developed.[56, 57] At basic level, the claim that herbal medicines are useful to treat IBD is often exaggerated. Most of the studies are performed using plant extracts without any chemical characterization and conclusions are often based on results of *in vitro* experiments to evaluate antioxidant or anti-inflammatory activity. When animal studies are used, researchers often employ a single acute model of rodent IBD, frequently chemically induced (TNBS/DSS). Similarly, clinical investigations have serious drawbacks regarding number of patients, short time frames of administration without long-term follow- up, lack of placebo group, standardized scores for endoscopic and histologic evaluation, and poor biochemical characterization. In agreement, Meta-analysis by Rahimi *et al* showed that herbal medicines may induce clinical efficacy in patients with IBD, but the evidence is too limited to make any confident conclusion.[58] As a result, the application of medicinal plants to treat IBD is significantly restricted.[55]

2.2 *Physalis peruviana* L.

2.2.1 Botanical description

Physalis is a genus of herbaceous plants in the Solanaceae family. The genus comprises about 120 species distributed throughout the tropical and subtropical regions of the world, naturally found in America, with a few introduced species in Europe and the countries of the Southwest and Center Asia.[59, 60] Typical *Physalis* species are herbaceous and possess solitary flowers, a yellow corolla, and an accrescent and inflated fruiting calyx.[59]

Physalis peruviana L. [Synonyms: *Alkekengi pubescens* Moench, *Boberella peruviana* (L.) E.H.L. Krause, *Physalis esculenta* Salisbury, *Physalis latifolia* Lam., *Physalis peruviana* var. latifolia (Lam.) Dunal, *Physalis tomentosa* Medik][61], is probably one of the best known species of this botanical genus due to its economic importance.[62] This plant is indigenous to South American Andes, in the high altitude of tropical Colombia, Chile, Ecuador and Peru, where the plant grows wild. Currently it can be found in tropical and subtropical regions including Malaysia, China and the Caribbean, where is grown from sea level (New Zealand) to 3000 m. elevations (Andes).[63]

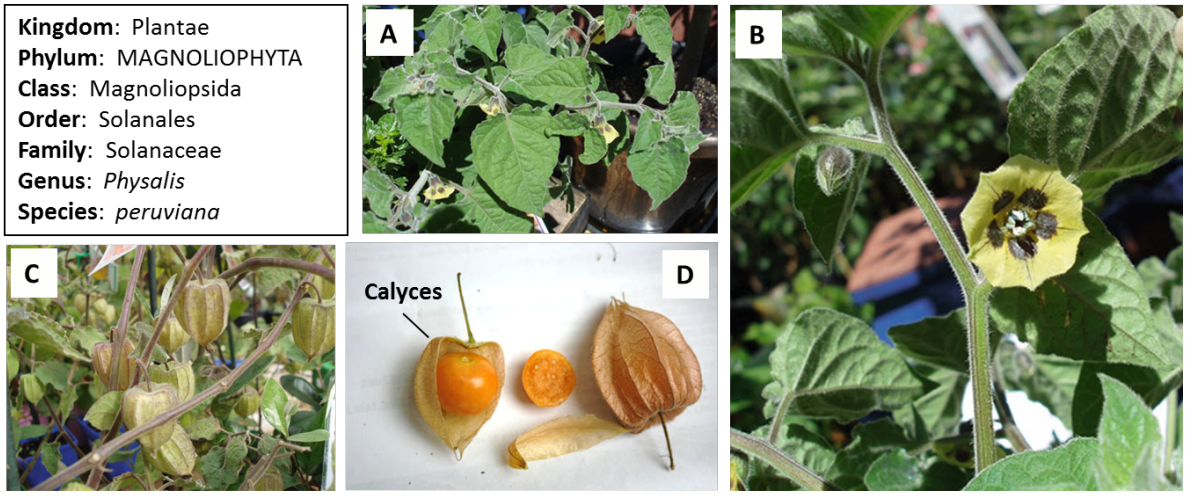


Figure 2.3. Images of *Physalis peruviana* L. The upper part describes the complete taxonomical classification of cape gooseberry/uchuva (*P. peruviana*). Pictures of (A.) Leaves and flowers; (B.) Close-up of flowers; (C.) Fruits enclosed in the parchment-like husk (calyces); and (D.) Fruit with calix removed. Adapted from [63].

P. peruviana is an herbaceous or soft-wooded, semi-shrub, upright, perennial plant, profusely branched, with densely pubescent and ribbed, often purplish branches. In natural, non-managed conditions, plant is known to reach 1.0 to 1.5 m in height.[64] The vegetative stage is characterized by the occurrence of simple, petiolated, alternate, heart-shaped leaves that are densely pubescent, with dimensions ranging from 5 to15 cm long and 4 to 10 cm wide.[64] Flowers (1.2-1.5 × 1.2-2 cm) occur in leaf axils on 1.5 cm pedicel with bell-shaped purplish-green pubescent calyx, yellow corolla and purple spotted in throat.[63] The

flower can be easily pollinated by insects, wind and also by auto-pollination. After fertilization, the calyx (or husk), which is small at the beginning of fruit development, grows to a bladder-like, papery organ, which completely encloses the ripening fruit.[64, 65] This unique structure protects the fruit against insects, birds, disease and adverse climatic situations. Moreover calyces represent an essential source of carbohydrates during the first 20 days of growth and development.[65] The fruit is a juicy berry, 1.25-2 cm diameter that turn from green to yellow upon ripening, with numerous flat seeds (2 mm diameter) embedded in the pulp.[63] When fully ripe the fruit is sweet but with a pleasant grape-like tang. The calyx is bitter and inedible.

2.2.2 Ethnopharmacology

Most species of the genus *Physalis* have been used for a long time in the medicinal folk traditions of Asian and American populations to treat different illnesses, such as malaria, asthma, hepatitis, dermatitis, rheumatism, liver disorders, and as an anti-mycobacterial, anticancer, anti-leukemic, antipyretic, and immunomodulatory agents.[60]

P. peruviana is a valued medicinal plant widely used in folk medicine of South America and Africa. The traditional uses reported for this species are presented in Table 2.1.

Table 2.1 Traditional Medicinal uses of *Physalis peruviana* L.

Part of the plant	Traditional Use	Preparation	Country	Ref
Leaves	Diuretic and anti-asthmatic	Decoction	Colombia	[66-71]
	Inflammation	Poultice	South Africa	
	Enema for abdominal ailments	Infusion	Southern Africa	
	Parasites and bowel complaints	Juice	South Africa	
	Diarrhea	Decoction	Kenya	
	Diabetes, Malaria, Pneumonia	Decoction	Tanzania	
	Skin fungal infections	Juice	Uganda	
	Labor Inducer	Juice		
Flower	Cough medicine	Decoction	Colombia	[72-74]
	Skin infections/problems.	Decoction	Ecuador	
	Promote wound healing		Uganda	
Calyces	Inflammation Cancer Diuretic	Poultice	Colombia	[75]
Fruit	Pterygium, Cataracts	Juice	Colombia	[65, 72, 76, 77]
	Albuminuria		Peru	
	Diabetes	Daily intake		

2.2.3 Anti-inflammatory activity

The majority of bioassay data describing the pharmacological activity of *P. peruviana* L. indicates an important antimicrobial, cytotoxic, antioxidant and anti-inflammatory effect. This section focuses on the studies related to inflammation.

Wu *et al* reported that a supercritical carbon dioxide extract from *P. peruviana* leaves (SCEPP-5; 30 μg/mL) possessed a strong inhibitory activity in lipopolysaccharide (LPS)-activated RAW264.7 cells, reducing nitric oxide (NO) and prostaglandin E2 (PGE2) levels through attenuation of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression.[78] Recently, Sang-ngern *et al* described the isolation of 4β-hydroxywhitanolide E from aerial parts of *P. peruviana*, which inhibited TNF-α induced NF-κB activity in stably transfected human embryonic kidney cells NF-κB Luc293. This effect correlated with a strong inhibition of NO production by LPS-induced RAW264.7 macrophages.[79]

The bioactivity of *P. peruviana* fruit juice has also been studied. Pardo *et al* found a mild anti-inflammatory effect of the juice in a rabbit eye inflammatory model. In addition, authors established a concentration-dependent cytostatic effect on cultured fibroblasts. Overall, the evidence might correlate with the traditional employment of *P. peruviana* to treat pterygium.[76]

In agreement with the traditional employment of *P. peruviana* calyces, several studies have demonstrated a strong anti-inflammatory activity *in vivo* and *in vitro*. The ethereal extract and various fractions from *P. peruviana* calyces were found to potentially inhibit the ear edema induced by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Authors highlighted the activity of fraction Pp-D28-LF, which demonstrated a dose-dependent activity as well as easy isolation and good yield.[75] In another study, this fraction was shown to exert a 40% inhibition of the acetic acid induced writhing (acute visceral nociception) and the licks during phase II (acute inflammatory nociception) of the formalin test; which suggests that anti-nociceptive effect of *P. peruviana* calyces might be related to NSAID-like mechanisms.[80] A complementary work from the same research team, showed the bioguided isolation of rutin and nicotoflorin from the ethanolic extract of *P. peruviana* calyces, using TPA-induced ear edema.[81]

Furthermore, a strong immunomodulatory effect of ethereal extract and fractions from *P. peruviana* calyces was demonstrated by Martinez *et al* when studying *Leishmania panamensis* infection to murine macrophages. In this study, extracts and fractions increased the proportion of infected cells with parasites by down-regulating macrophage activation, and a strong inhibition of pro-inflammatory cytokines (IL6, TNF-α, and MCP-1).[82]

2.3 References

1. Hisamatsu, T., et al., *Immune aspects of the pathogenesis of inflammatory bowel disease.* Pharmacology & Therapeutics, 2013. **137**(3): p. 283-297.
2. Louis, E., C. Van Kemseke, and C. Reenaers, *Necessity of phenotypic classification of inflammatory bowel disease.* Best Pract Res Clin Gastroenterol, 2011. **25 Suppl 1**: p. S2-7.
3. Fiocchi, C., *IBD: advances in pathogenesis, complications, diagnosis, and therapy.* Curr Opin Gastroenterol, 2012. **28**(4): p. 297-300.
4. Castiglione, F., et al., *Risk factors for inflammatory bowel diseases according to the "hygiene hypothesis": a case-control, multi-centre, prospective study in Southern Italy.* J Crohns Colitis, 2012. **6**(3): p. 324-9.
5. Sartor, R.B., *Mechanisms of Disease: pathogenesis of Crohn's disease and ulcerative colitis.* Nat Clin Pract Gastroenterol Hepatol, 2006. **3**(7): p. 390-407.
6. Zhang, Y.Z. and Y.Y. Li, *Inflammatory bowel disease: pathogenesis.* World J Gastroenterol, 2014. **20**(1): p. 91-9.
7. Molodecky, N.A., et al., *Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review.* Gastroenterology, 2012. **142**(1): p. 46-54
8. Cosnes, J., et al., *Epidemiology and natural history of inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1785-94.
9. Ng, S.C., et al., *Incidence and phenotype of inflammatory bowel disease based on results from the Asia-pacific Crohn's and colitis epidemiology study.* Gastroenterology, 2013. **145**(1): p. 158-165.
10. Burisch, J. and P. Munkholm, *Inflammatory bowel disease epidemiology.* Curr Opin Gastroenterol, 2013. **29**(4): p. 357-62.
11. Lovasz, B.D., et al., *New trends in inflammatory bowel disease epidemiology and disease course in Eastern Europe.* Dig Liver Dis, 2013. **45**(4): p. 269-76.
12. Wong, S. and S. Ng, *What Can We Learn From Inflammatory Bowel Disease in Developing Countries?* Current Gastroenterology Reports, 2013. **15**(3): p. 1-9.
13. Gismera, C. and B. Aladren, *Inflammatory bowel diseases: a disease (s) of modern times? Is incidence still increasing?* World J Gastroenterol, 2008. **14**(36): p. 5491-8.
14. Pineda, L., *Enfermedad inflamatoria intestinal en Colombia: ¿Está cambiando nuestro perfil epidemiológico?* Revista Colombiana de Gastroenterologia, 2010. **25**: p. 235-238.
15. Juliao, F., et al., *Fenotipo e historia natural de la enfermedad inflamatoria intestinal en un centro de referencia en Medellín-Colombia.* Revista Colombiana de Gastroenterologia, 2010. **25**: p. 240-251.
16. Neurath, M.F., *Cytokines in inflammatory bowel disease.* Nat Rev Immunol, 2014. **14**(5): p. 329-342.
17. Dave, M., K.A. Papadakis, and W.A. Faubion, Jr., *Immunology of inflammatory bowel disease and molecular targets for biologics.* Gastroenterol Clin North Am, 2014. **43**(3): p. 405-24.
18. de Souza, H.S. and C. Fiocchi, *Immunopathogenesis of IBD: current state of the art.* Nat Rev Gastroenterol Hepatol, 2016. **13**(1): p. 13-27.
19. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nat Rev Immunol, 2014. **14**(3): p. 141-153.
20. Konig, J., et al., *Human Intestinal Barrier Function in Health and Disease.* Clin Trans Gastroenterol, 2016. **7**: p. e196.
21. Maloy, K. and F. Powrie, *Intestinal homeostasis and its breakdown in inflammatory bowel disease.* Nature, 2011. **474**(7351): p. 298-306.
22. Petersson, J., et al., *Importance and regulation of the colonic mucus barrier in a mouse model of colitis.* Am J Physiol Gastrointest Liver Physiol, 2011. **300**(2): p. G327-33.

23. Abreu, M.T., *Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function.* Nat Rev Immunol, 2010. **10**(2): p. 131-44.
24. Hausmann, M., et al., *Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation.* Gastroenterology, 2002. **122**(7): p. 1987-2000.
25. Fukata, M., et al., *Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis.* Inflamm Bowel Dis, 2011. **17**(7): p. 1464-73.
26. Rakoff-Nahoum, S., et al., *Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis.* Cell, 2004. **118**(2): p. 229-241.
27. Gibson, D., et al., *MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during Citrobacter rodentium-induced colitis.* Cell Microbiol, 2008. **10**(3): p. 618-31.
28. Scharl, M. and G. Rogler, *Microbial sensing by the intestinal epithelium in the pathogenesis of inflammatory bowel disease.* Int J Inflam, 2010. **2010**: p. 671258.
29. Khor, B., A. Gardet, and R.J. Xavier, *Genetics and pathogenesis of inflammatory bowel disease.* Nature, 2011. **474**(7351): p. 307-17.
30. Saleh, M. and C.O. Elson, *Experimental inflammatory bowel disease: insights into the host-microbiota dialog.* Immunity, 2011. **34**(3): p. 293-302.
31. Fritz, T., et al., *Crohn's disease: NOD2, autophagy and ER stress converge.* Gut, 2011. **60**(11): p. 1580-8.
32. Wehkamp, J., et al., *Reduced Paneth cell alpha-defensins in ileal Crohn's disease.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18129-34.
33. Clevers, H.C. and C.L. Bevins, *Paneth cells: maestros of the small intestinal crypts.* Annu Rev Physiol, 2013. **75**: p. 289-311.
34. Negroni, A., et al., *NOD2 induces autophagy to control AIEC bacteria infectiveness in intestinal epithelial cells.* Inflamm Res, 2016. **65**(10): p. 803-13.
35. Cadwell, K., et al., *A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells.* Nature, 2008. **456**(7219): p. 259-63.
36. Kaser, A., L. Niederreiter, and R. Blumberg, *Genetically determined epithelial dysfunction and its consequences for microflora-host interactions.* Cell Mol Life Sci, 2011. **68**(22): p. 3643-9.
37. Kaser, A., et al., *XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease.* Cell, 2008. **134**(5): p. 743-56.
38. Martinon, F., et al., *TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages.* Nat Immunol, 2010. **11**(5): p. 411-8.
39. Xavier, R.J. and D.K. Podolsky, *Unravelling the pathogenesis of inflammatory bowel disease.* Nature, 2007. **448**(7152): p. 427-434.
40. Maynard, C. and C. Weaver, *Intestinal effector T cells in health and disease.* Immunity, 2009. **31**(3): p. 389-400.
41. Geremia, A., et al., *Innate and adaptive immunity in inflammatory bowel disease.* Autoimmun Rev, 2014. **13**(1): p. 3-10.
42. Jostins, L., et al., *Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease.* Nature, 2012. **491**(7422): p. 119-24.
43. Bogaert, S., et al., *Differential mucosal expression of Th17-related genes between the inflamed colon and ileum of patients with inflammatory bowel disease.* BMC Immunology, 2010. **11**(1): p. 61.
44. Cho, J.H., *The genetics and immunopathogenesis of inflammatory bowel disease.* Nat Rev Immunol, 2008. **8**(6): p. 458-66.
45. Budarf, M., et al., *GWA studies: rewriting the story of IBD.* Trends Genet, 2009. **25**(3): p. 137-46.

46. Abraham, C. and J. Cho, *Interleukin-23/Th17 pathways and inflammatory bowel disease.* Inflamm Bowel Dis, 2009. **15**(7): p. 1090-100.
47. Grant, C.R., et al., *Regulatory T-cells in autoimmune diseases: Challenges, controversies and—yet—unanswered questions.* Autoimmunity Reviews, 2015. **14**(2): p. 105-116.
48. Geremia, A., et al., *Innate and adaptive immunity in inflammatory bowel disease.* Autoimmunity Reviews, 2014. **13**(1): p. 3-10.
49. Mayne, C.G. and C.B. Williams, *Induced and natural regulatory T cells in the development of inflammatory bowel disease.* Inflamm Bowel Dis, 2013. **19**(8): p. 1772-88.
50. Ferrara, L. and S. Saccomano, *Crohn disease: Recognition is key.* Nurse Pract, 2012. **37**(12): p. 22-8.
51. Baumgart, D.C. and W.J. Sandborn, *Inflammatory bowel disease: clinical aspects and established and evolving therapies.* The Lancet. **369**(9573): p. 1641-1657.
52. de Mattos, B.R., et al., *Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments.* Mediators Inflamm, 2015. **2015**: p. 493012.
53. Zabana, Y., et al., *Infliximab safety profile and long-term applicability in inflammatory bowel disease: 9-year experience in clinical practice.* Aliment Pharmacol Ther, 2010. **31**(5): p. 553-60.
54. Strong, S., *Inflammatory bowel disease surgery in the biologic therapy era.* Curr Opin Gastroenterol, 2012. **28**(4): p. 349-53.
55. Rahimi, R., S. Mozaffari, and M. Abdollahi, *On the Use of Herbal Medicines in Management of Inflammatory Bowel Diseases: A Systematic Review of Animal and Human Studies.* Digestive Diseases and Sciences, 2009. **54**(3): p. 471-480.
56. Debnath, T., D.H. Kim, and B.O. Lim, *Natural products as a source of anti-inflammatory agents associated with inflammatory bowel disease.* Molecules, 2013. **18**(6): p. 7253-70.
57. Hur, S.J., et al., *Review of natural products actions on cytokines in inflammatory bowel disease.* Nutr Res, 2012. **32**(11): p. 801-16.
58. Rahimi, R., S. Nikfar, and M. Abdollahi, *Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: a meta-analysis.* World J Gastroenterol, 2013. **19**(34): p. 5738-49.
59. Zamora-Tavares, M.d.P., et al., *Physalis and physaloids: A recent and complex evolutionary history.* Molecular Phylogenetics and Evolution, 2016. **100**: p. 41-50.
60. Zhang, W.N. and W.Y. Tong, *Chemical Constituents and Biological Activities of Plants from the Genus Physalis.* Chem Biodivers, 2016. **13**(1): p. 48-65.
61. The Plant List. *Version 1.1*. 2013; Available from: http://www.theplantlist.org/.
62. Samuels, J., *Biodiversity of Food Species of the Solanaceae Family: A Preliminary Taxonomic Inventory of Subfamily Solanoideae.* Resources, 2015. **4**(2): p. 277.
63. Lim, T.K., *Physalis peruviana*, in *Edible Medicinal And Non-Medicinal Plants*2013, Springer Netherlands. p. 300-309.
64. Ramírez, F., et al., *Cape gooseberry (Physalis peruviana L.) phenology according to the BBCH phenological scale.* Scientia Horticulturae, 2013. **162**: p. 39-42.
65. Puente, L.A., et al., *Physalis peruviana Linnaeus, the multiple properties of a highly functional fruit: A review.* Food Research International, 2011. **44**(7): p. 1733-1740.
66. Morton, J.F., *Cape Gooseberry - Physalis peruviana*, in *Fruits of Warm Climates*, C.R. Systems, Editor 1987: Miami, FL. p. 5-6.
67. Ahmad, S., et al., *Withanolides from Physalis peruviana.* Phytochemistry, 1999. **50**(4): p. 647-651.
68. Madikizela, B., et al., *Ethnopharmacological study of plants from Pondoland used against diarrhoea.* J Ethnopharmacol, 2012. **141**(1): p. 61-71.
69. Maobe, M.A.G., et al., *Antimicrobial activities of eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in kisii region,southwest Kenya.* Global Journal of Pharmacology, 2013. **7**(1): p. 25-33.

70. Runyoro, D.K.B., et al., *Medicinal plants used by Tanzanian traditional healers in the management of Candida infections.* Journal of Ethnopharmacology, 2006. **106**(2): p. 158-165.
71. Kamatenesi-Mugisha, M. and H. Oryem-Origa, *Medicinal plants used to induce labour during childbirth in western Uganda.* Journal of Ethnopharmacology, 2007. **109**(1): p. 1-9.
72. Ospina, L.F. and R. Pinzon, *Plantas usadas como antidiabeticas en la medicina popular Colombiana.* Revista Colombiana de Ciencias Químico-Farmacéuticas, 1995(23): p. 81-94.
73. Tene, V., et al., *An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador.* Journal of Ethnopharmacology, 2007. **111**(1): p. 63-81.
74. Namukobe, J., et al., *Traditional plants used for medicinal purposes by local communities around the Northern sector of Kibale National Park, Uganda.* Journal of Ethnopharmacology, 2011. **136**(1): p. 236-245.
75. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica: revista del Instituto Nacional de Salud, 2007. **27**(1): p. 110.
76. Pardo, J.M., et al., *Determining the pharmacological activity of Physalis peruviana fruit juice on rabbit eyes and fibroblast primary cultures.* Invest Ophthalmol Vis Sci, 2008. **49**(7): p. 3074-9.
77. Rodríguez, S. and E. Rodríguez, *Efecto de la ingesta de Physalis peruviana (aguaymanto) sobre la glicemia postprandial en adultos jóvenes.* Revista Médica Vallejiana, 2007. **4**(1): p. 43-52.
78. Wu, S.J., et al., *Supercritical carbon dioxide extract exhibits enhanced antioxidant and anti-inflammatory activities of Physalis peruviana.* Journal of Ethnopharmacology, 2006. **108**(3): p. 407-413.
79. Sang-ngern, M., et al., *Withanolides derived from Physalis peruviana (Poha) with potential anti-inflammatory activity.* Bioorganic & Medicinal Chemistry Letters, 2016. **26**(12): p. 2755-2759.
80. Muñoz, C.E., et al., *Antinociceptive effect of Critoniella acuminata, Physalis peruviana and Salvia rubescens.* Revista Colombiana de Ciencias Químico-Farmacéuticas, 2009. **38**(1): p. 31-41.
81. Toro, R.M., et al., *Phytochemical analysis, antioxidant and anti-inflammatory activity of calyces from Physalis peruviana.* Nat Prod Commun, 2014. **9**(11): p. 1573-5.
82. Martinez, W., et al., *In vitro studies on the relationship between the anti-inflammatory activity of Physalis peruviana extracts and the phagocytic process.* Immunopharmacol Immunotoxicol, 2010. **32**(1): p. 63-73.

3 CHAPTER THREE. Sucrose esters from *Physalis peruviana* calyces with anti-inflammatory activity.

Luis A. Franco[1], Yanet C. Ocampo[1], Harold A. Gómez[1], Rocío De la Puerta[2], José L. Espartero[3], Luis F. Ospina[4]

[1]Biological Evaluation of Promising Substances Group, Faculty of Pharmaceutical Sciences, University of Cartagena, Cartagena, Colombia.
[2] Pharmacology Department, Faculty of Pharmacy, University of Seville, Seville, Spain.
[3] Organic Chemistry Department, Faculty of Pharmacy, University of Seville, Seville, Spain
[4] Pharmacy Department, Faculty of Sciences, National University of Colombia, Bogotá, Colombia.

3.1 Abstract

Physalis peruviana L. is a native plant from the South American Andes that is widely used in the traditional medicine of Colombia as anti-inflammatory medicinal plant, specifically the leaves, calyces, and small stems in poultice form. Previous studies performed by our group on *P. peruviana* calyces showed potent anti-inflammatory activity in an enriched fraction obtained from an ether total extract. The objective of the present study was to obtain and elucidate the active compounds from this fraction and evaluate their anti-inflammatory activity *in vivo* and *in vitro*. The enriched fraction of *P. peruviana* was purified by several chromatographic methods to obtain a mixture of two new sucrose esters named peruviose A **(1)** and peruviose B **(2)**. Structures of the new compounds were elucidated using spectroscopic methods and chemical transformations. Acute toxicity was assessed to define the dosage levels for bioactivity evaluation. The anti-inflammatory activity of mixture of peruvioses was evaluated using λ-carrageenan-induced paw edema in rats and LPS-activated peritoneal macrophages. Results showed that peruvioses have toxic effects at doses above 200 mg/Kg without producing toxicity on liver and kidney. On the other hand, peruvioses significantly attenuated the inflammation induced by λ-carrageenan in a dosage-dependent manner, probably due to an inhibition of NO• and PGE2, which was demonstrated *in vitro*. To our knowledge, this is the first report of the presence of sucrose esters in *P. peruviana*, which showed potent anti-inflammatory effect. These results suggest the potential of sucrose esters from *Physalis* genus as a novel natural alternative to treat inflammatory diseases.

Key words: Sucrose esters, *Physalis peruviana*, Solanaceae, NO, PGE2, TNF-α

3.2 Introduction

Inflammation is a frequent and immediate response to external and internal injurious stimuli including: infections, chemicals, physical stress, and immune pathologies. It is a complex phenomenon that involves many cell types and cellular pathways.[1] Molecules

produced during inflammation trigger pain receptors, induce local vasodilatation and recruit phagocytic cells, especially neutrophils and macrophages, which then activate other immune system components.[2] Excessive or persistent inflammation leads to overproduction of mediators, that are a key factor for the development of severe pathologies such as rheumatoid arthritis [3], bacterial sepsis [4], asthma [5], atherosclerosis [6], inflammatory bowel disease [7], and cancer [8], which represent an important cause of morbidity worldwide. Thus, reduction or elimination of the persistent inflammatory response and/or the overproduction of pro-inflammatory mediators, is an important target to prevent or treat these diseases.[9]

Plants represent an excellent source to obtain new drugs, as they constitute an immense reservoir of structurally diverse secondary metabolites that might potentially inhibit the inflammatory process by affecting different molecular targets.[4] In this sense, *Physalis peruviana* L. (Solanaceae) widely used in traditional medicine as a diuretic and hypoglycemic agent and to treat malaria, asthma, hepatitis, dermatitis, rheumatoid arthritis [10], represents a promissory source of bioactive compounds. This has been experimentally validated, with extracts showing relevant antioxidant [11, 12], antiproliferative [13-15], cytotoxic [16], anti-hepatotoxic [17], hypoglycemic [18], immunomodulatory [19], and anti-inflammatory activities [20, 21].

P. peruviana also known as “uchuva” in Colombia or “gooseberry” in English speaking countries, is native from tropical South America and characterized because the fruit grows enclosed in a papery husk or calyx, which is one of the best-known examples of persistent sepals due to its conspicuous post-floral growth and enlargement.[22-24] *P. peruviana* calyx protects the fruit against insects, birds, diseases, and adverse climatic situations; this structure represents an essential source of carbohydrates during the first 20 days of growth and development of the fruit, and conserve it even after harvest, allowing a shelf life of one month with it, whereas without calyx is 4 to 5 days or so.[23, 24] Three varieties of *P. peruviana* are currently grown, originating from Colombia, Kenya and South Africa. The ecotype Colombia, which has a great demand on the international markets, presents small and colorful fruits and different morphological characteristics in the calyx.[25] Although the fruit is highly appreciated for its commercial value, the calyx constitutes a waste generated in fruit production as well as an unexplored source of bioactive molecules.

The experimental studies in *P. peruviana* have been focused in the chemical and biological characterization of the whole plant, stems, leaves, and fruits, with only few reports for calyces. Chemical studies on *P. peruviana*, mainly in the aerial parts, showed presence of withanolides, steroids, alkaloids, and glycosides.[15-17, 23, 26] Phytochemical studies made in our research group on *P. peruviana* calyces indicated presence of flavonoids, steroids and/or triterpenes, and lactones α-β unsaturated. Our previous studies, also demonstrated that the major fraction obtained from the ether extract of *P. peruviana*

calyces, showed potent anti-inflammatory activity in a TPA-induced ear edema model, with inhibition nearly to 70%.[20] In this work we elucidated the structure of two new sucrose esters from this major fraction, and evaluated their anti-inflammatory activity *in vivo* in the λ-carrageenan-induced paw edema model and the *in vitro* effect on nitric oxide (NO•), prostaglandin E2 (PGE2), and tumor necrosis factor alpha (TNF-α) production from LPS-stimulated murine macrophages. Sucrose esters have been isolated from *Physalis* species in fruits, flowers, and stems.[27-29] To our knowledge, this is the first report on the presence of sucrose esters in calyces of *Physalis* genus.

3.3 Material and Methods

3.3.1 Experimental Instrumentation and Chemicals

Melting points were determined by Differential Scanning Calorimetry-DSC7 (Perkin-Elmer, USA) and are uncorrected. IR spectrum was recorded on a FTIR (Perkin Elmer 1600 series). NMR spectra were obtained on a Bruker AMX-500 spectrometer, with TMS as internal standard. FAB-MS were obtained on Kratos MS80-RFA mass spectrometer. A Hitachi-LaChrom Elite® apparatus equipped with photodiode array detector (PDA) was used for analytical HPLC separations. Reversed-phase chromatography was performed with a 100 x 4.6 mm Chromolith® C-18 column (Merck, Germany). TLC was performed on silica gel 60 F254 plates (250 μm thickness; Merck, Germany). Silica gel 60 (0.063-0.200 mm) and ammonium hydroxide (NH_4OH) were also obtained from Merck. All solvents were of analytical grade and purchased from Merck. A plethysmometer (model 7140 Ugo Basile, Italy) was used to determine paw volume in rats.

3.3.2 Plant Material

Calyces of *Physalis peruviana* L. were collected in La Mesa, Colombia (4º 37’ 49.22’’ N; 74º 27′ 45.60’’ W; elevation 1198 m.a.s.l.), in November 2003. Taxonomic identification was performed by Clara I. Orozco at Herbario Nacional Colombiano (Instituto de Ciencias Naturales, Universidad Nacional de Colombia), Bogotá, Colombia, where a voucher specimen (COL-512200) has been deposited.

3.3.3 Extraction and Isolation

Dried calyces (2 kg) were powdered and extracted with petroleum ether by percolation at room temperature, until exhaustion of the material. The concentrated extract (271.6 g) was partitioned with ether and methanol-water (9:1) to give a polar fraction (223.3 g). This fraction (22 g) was subjected to column chromatography (CC, 14x30 cm columns dimension) on silica gel (600 g, 0.063-0.200 mm) and eluted with petroleum ether, dichloromethane, ethyl acetate, and methanol mixtures gradually increasing polarity. Fractions were combined based on TLC examination using a proper mobile phase and visualized by heating after spraying with Godin reagent [30] to obtain 38 main fractions with a 94.77% of efficiency. The major fraction (8.23 g), named Pp-D_{28}-LF [20], was

subjected to analytical HPLC analysis using a mixture of methanol and 0.07M monobasic potassium phosphate buffer (6.5:3.5, pH 4.0) as mobile phase, 1 mL/min flow rate and operating temperature of 25 °C; to show one component that constituted nearly 82% of the mixture. This major fraction (2 g) was purified by CC (gradient elution CH_2Cl_2 to EtOAc) followed by two successive preparative TLCs (eluent CH_2Cl_2-EtOAc 4:6) to yield 293 mg of an inseparable mixture of two new sucrose esters: Peruviose A **(1)** and Peruviose B **(2)**. Their structures were elucidated trough IR, FAB-MS and extensive 2D NMR methods including, ^{1}H-NMR, ^{13}C-NMR, Dep90, Dep135, HMBC, and NOESY (Appendix 2).

Peruviose A and B (1 and 2). Light yellow gummy solid mixture of **(1)** and **(2)**; Rf 0.489 on silica gel 60 F-254 (CH_2Cl_2/EtOAc, 4:6); Purity (HPLC): 99.05% (t_R 2.35 min; CH_3OH/KH_2PO_3 (0.07 M), 6.5:3.5, pH 4.0); mp 20-22 °C (uncorrected); UV (EtOH) λmax 220 nm; $[\alpha]_D^{20°C}$=+52.08, IR (KBr) ν_{max} 3411, 2927, 2858, 1746, 1191, 1155, 1063, 1017 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR, HMBC and NOESY (125 MHz, $CDCl_3$) spectra. FAB-MS: **(1)** *m/z* 729 $[M+Na]^+$ (calcd. for $C_{34}H_{58}O_{15}Na$), *m/z* 233 $[C_{10}H_{17}O_6]$, *m/z* 155 $[C_{10}H_{19}O]$, and *m/z* 71 $[C_4H_7O]$; **(2)** *m/z* 743 $[M+Na]^+$, (calcd. for $C_{35}H_{60}O_{15}Na$), *m/z* 247 $[C_{11}H_{19}O_6]$, *m/z* 155 $[C_{10}H_{19}O]$, *m/z* 85 $[C_5H_9O]$, and *m/z* 71 $[C_4H_7O]$.

3.3.4 Chemical modifications

To verify the presence of glycosylated esters and the absolute configuration of the sugar moiety, the mixture of peruviose A and B (10 mg) was hydrolyzed with 2 mL of NH_4OH 2M for 4 hours at 50 ºC. The reaction mixture was adjusted to pH 3 by addition of formic acid 2M, and subjected to successive liquid-liquid extraction with ethyl acetate (3x3 mL). The aqueous solution was used to determine the absolute configuration of glycosides. Additionally, 90 mg the mixture of compounds were acetylated by usual procedure with acetic anhydride and pyridine (5:1 per gram of the compound), to yield 90.9 mg of the acetylated material (efficiency 81.9%), after usual work-up. Acetylated peruviose A and B **(3** and **4)**, were submitted to ^{1}H-NMR, ^{13}C-NMR, Dep90, Dep135, HMBC, NOESY and FABMS, to confirm the elucidation of their structure.

Acetylated peruviose A and B. Light yellow gummy solid; NMR (500 MHz, $CDCl_3$) FAB-MS: *m/z* 897 $[M+Na]^+$ (calcd. for $C_{42}H_{66}O_{19}Na$), and *m/z* 911 $[M+Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$).

3.3.5 Experimental animals

Female Wistar rats (140-170 g) and ICR mice (20-25 g) were provided by Instituto Nacional de Salud, Colombia. Animals were allowed to acclimatize for 10 days before use and fed with standard rodent food and water *ad libitum*. They were housed in filtered-capped polycarbonate cages and kept in a controlled environment at 22±3 °C and relative humidity between 65 to 75%, under a cycle of 12 h light/darkness. Animals were sacrificed by cervical dislocation at the corresponding time of each experiment. All experiments were

designed and conducted in accordance with the guidelines of the Ethics Committee of University of Cartagena (Minutes of October 14 of 2010, Appendix 3) and the European Union regulations (CEC council 86/809).

3.3.6 Acute toxicity

The acute toxicity test was carried out to evaluate any possible toxic effect exerted by the mixture of peruviose A and B from *P. peruviana* calyces. Mice were randomly divided into ten groups of six animals per group, and treated intraperitoneally with graded doses of sucrose esters ranging from 0 (control) to 300 mg/Kg. Mice were observed for 24 h post-treatment for mortality, behavioral changes and signs of toxicity. Lethal dose 50 (LD_{50}) value was determined by the Miller and Tainter method.[31] At the end of experiment, all animals were sacrificed and kidney and liver were carefully excised for histological examination, fixed in 4% buffered formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin using standard techniques. All slides were coded and evaluated in a blinded manner by a pathologist observer according to the parameters described in Appendix 4.

3.3.7 λ-Carrageenan paw edema

The anti-inflammatory activity was evaluated according to the method described by Winter *et al.*[32] Edema was induced by sub-plantar injection of λ-carrageenan (0.1 mL of a 2% solution) in the right hind paw of each animal. Rats were randomly divided into five groups of six animals each, and Saline (Control), Indomethacin 10 mg/Kg (positive control), or the mixture of peruviose A and B (100, 50 and 25 mg/kg) was prophylactically administered (i.p.), 1 h before administration of λ-carrageenan. Paw volume was determined by means of a volume displacement method using a plethysmometer immediately prior to the injection of λ-carrageenan and thereafter 1, 3, and 5 h. Edema was expressed as the increase in paw volume (mL) after λ-carrageenan injection relative to the pre-injection value for each animal.

3.3.8 MTT assay

The mitochondrial-dependent reduction of MTT to formazan was used to assess the cytotoxic effect of test compounds.[33, 34] Cells ($1x10^6$ cells/mL) were cultured at 37°C with various concentrations of peruviose A and B (0.1-100 μg/mL). Triton X-100 (20 %) was used as positive control. After 24 h, the medium was removed and cells incubated with MTT solution (3 mg/mL). Four hours later, the medium was carefully aspirated and formazan crystals were dissolved in DMSO (100 μL). OD_{550} was measured using a microplate reader (Multiskan EX Thermo®).

3.3.9 NO production

NO• release was determined spectrophotometrically by the accumulation of nitrite (NO_2^-), stable metabolite of the reaction of NO• with oxygen, using the Griess reaction [35].

Briefly, 100 µL of cell culture supernatant was mixed with 100 µL of Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4), and incubated at room temperature for 5 min. The OD_{550} of the samples was measured using a microplate reader and compared with a standard curve prepared with $NaNO_2$ (1-200 µM). Additionally, a direct NO•-scavenging effect of peruviose A and B, was determined as described in Supporting Information.

3.3.10 TNF-α and PGE2 release

Levels of TNF-α and PGE2 in culture supernatants were determined using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Final results were expressed as ng/mL.

3.3.11 Statistical Analysis

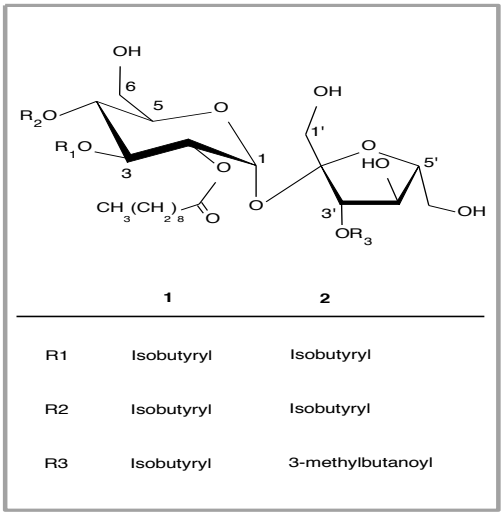
Data are presented as Mean ± Standard Error of the Mean (SEM). Concentrations that inhibited cell survival (LC_{50}) and inflammatory mediators (IC_{50}) by 50% were calculated employing non-linear regression. Statistical comparisons between groups were established using analysis of variance (ANOVA), followed by Dunnett test *post hoc* analysis. *P* values less than 0.05 were considered statistically significant.

3.4 Results and Discussion

Colum chromatographic purification of the methanol-water soluble fraction, obtained by liquid/liquid partition from the total ether extract of *P. peruviana* calyces, provided a major fraction which constituted 3.03% of the initial material and was subjected to preparative TLC to lead the isolation of one spot that showed a single peak on reversed-phase HPLC. However, spectroscopic signals of this fraction revealed a mixture of two new sucrose esters: Peruviose A **(1)** and B **(2)**, occurring in a 6:4 ratio, respectively. All attempts at the separation of the components with several combinations of solvent systems using preparative TLC and HPLC were ineffective. Therefore, we elucidate their structures by analyzing the spectroscopic data of the mixture.

^{1}H-NMR and ^{13}C-NMR signals and the connectivities observed in the HMBC and NOESY spectra for **(1)**, revealed the presence of one pyranose and one furanose units as the monomers of a disaccharide structure, as well as four acyl substituents: three isobutanoyl and one decanoyl chains (Table 3.1 and Figure 3.1). The relative configuration of the sugars was determined using comparisons of ^{1}H-NMR coupling constants and ^{13}C-NMR chemical shifts with literature values.[26-29] The pyranose was identified as α-D-glucopyranose by the observed coupling constants ($J_{1,2}$ = 3.7 Hz, $J_{2,3}$ = 10.4 Hz, $J_{3,4}$ = 9.4 Hz) that also established H-1 as equatorial, whereas the strong coupling observed between H-2 and H-3, H-3 and H-4, and H-4 and H-5 established these protons as axial. On the other hand, the furanose was identified as β-D-fructofuranose showing the following coupling constants ($J_{3',4'}$(H3′)=$J_{3',4'}$(H4′)=8.2 Hz). The NOE contact between glucose H-1

to H-1a´ and H-1b´ of the fructose ring, confirmed the glycosidic linkage with α- and β-orientation on the anomeric carbon for D-glucose and D-fructose, respectively. Moreover, HMBC signal between H-1 and C-2´ unequivocally indicates the 1,2 linkage between D-glucose and D-fructose.



	1	2
R1	Isobutyryl	Isobutyryl
R2	Isobutyryl	Isobutyryl
R3	Isobutyryl	3-methylbutanoyl

Figure 3.1. Structure of Peruviose A (1) and B (2) isolated from calyces of *Physalis peruviana*.

Compounds **(1)** and **(2)** were isolated as a gummy solid, 99.05% purity based on HPLC, that exhibited in the IR spectrum strong absorption bands for hydroxyl (ν=3411 cm^{-1}) and saturated ester functions (ν=1746 cm^{-1}). Despite the fact that it was a mixture, NMR spectra displayed many duplicated peaks slightly displaced and MS showed similar but distinguishable fragmentation pathways, which allowed the elucidation of both compounds separately.

The positions of all substituents in the disaccharide structure were determined from the analysis of the HMBC spectrum, which showed correlations between H-2 and the carboxylic carbon C-1''. This data evidences that the aliphatic decanoyl chain is placed in the position 2 of the glucose unit. On the other hand, HMBC spectrum showed correlations between H-3 of the glucopyranose and C-1''' of an isobutanoyl group, which in turn is coupled with H-3'''. The same bidimensional spectrum showed connectivities of H-4 with C-1'''' of another isobutanoyl group, which is coupled with H-3''''. In the furanose moiety a clear correlation between H-3' and carboxylic C-1''''' was found, indicating that another isobutanoyl substituent was located in position 3 of this unit.

This analysis led us to elucidate the structure of the new compound 2-*O*-decanoyl-3,3´,4-tri-*O*-isobutanoylsucrose named Peruviose A **(1)**, $C_{34}H_{58}O_{15}$. The adduct ion peak at *m/z* 729 $[M+Na]^+$ obtained by FABMS, as well as fragments at *m/z* 155 (decanoyl) and *m/z* 71 (isobutanoyl), are in agreement with the proposed molecular formula and structure. Additionally, the ion peak at *m/z* 897 $[M+Na]^+$ (calcd. for $C_{42}H_{66}O_{19}Na$) of the

corresponding acetylated derivate confirmed the presence of the four ester groups, while the fragment at *m/z* 233 supported the proposed substitution of the furanose moiety.

Table 3.1. NMR spectroscopic data for Peruviose A (1) in $CDCl_3$ (500 MHz for ^1H and 125 MHz for ^{13}C)

Number	^1H [δ(ppm), *m*, ^3J (Hz)]	^{13}C (δ, ppm)/DEPT	NOESY	HMBC
Glucose-1	5.59, *d, 3.7*	89.40/CH	H-2, H-1'a, H-1'b	C-5, C-2'
2	4.96-4.90 *	70.06/CH	H-1'a	C-3, C-1''
3	5.48, *dd, 10.4, 9.4*	69.02/CH	H-5	C-2, C-4, C-1'''
4	4.90-4.96 *	68.25/CH	H-6	C-3, C-5, C-6, C-1''''
5	4.12-4.19 *	72.18/CH	H-6	
6	3.60, *m*	61.60/CH_2	H-4'	
Fructose-1'a	3.58, *d, 12.0*	64.69/CH_2	H-3', H-5'	
1'b	3.45, *d, 12.0*		H-3'	
2'	—	103.81/C		
3'	5.20, *d, 8.2*	79.26/CH	H-5'	C-1', C-4', C-1'''''
4'	4.56, *t, 8.2*	71.19/CH	H-6'a, H-6'b	C-3', C-5', C-6'
5'	3.94, *m*	82.55/CH		C-4'
6'a	3.90, *m*	59.73/CH_2		C-4'
6'b	3.74, *m*			
1''	—	172,88/C=O		
2''	2.25, *m*	33.85/CH_2	H-3'', H-4''	C-3''
3''	1.54, *m*	24.55/CH_2	H-4''	C-2''
4''-7''	1.24, *s* wide	29.33-29.06/CH_2		
8''	1.24, *s* wide	31.82/CH_2		
9''	1.24, *s* wide	22.63/CH_2		
10''	0.87, *t, 7.0*	14.06/CH_3		C-8'', C-9''
1'''	—	175.74/C=O		
2'''	2.45, *m*, *7.0*	33.89/CH	H-3'''a, H-3'''b	
3'''a	1.07, *d*, *7.0*	18.79-18.70/CH_3		C-2'''
3'''b	1.08, *d*, *7.0*			C-2'''
1''''	—	175.98/C=O		
2''''	2.51, *m*, *7.0*	33.93/CH	H-3''''a, H-3''''b	C-3''''
3''''a	1.13, *d*, *7.0*	18.79-18.70/CH_3		C-2''''
3''''b	1.06, *d*, *7.0*			C-2''''
1'''''	—	177.87/C=O		
2'''''	2.75, *m*, 7.0	34.02/CH	H-3'''''a, H-3'''''b	C-3'''''
3'''''a	1.32, *d*, 7.0	18.87/CH_3		C-2'''''
3'''''b	1.29, *d*, 7.0	18.87/CH_3		C-2'''''

* The multiplicity could not be determined.

Compound **(2)** was identified on the basis of 1D and 2D NMR analysis and FABMS spectra in a similar manner. This analysis pointed out that both compounds shared an almost identical structure with an esterified sucrose core with four acyl groups (Table 3.2 and Figure 3.1). The only difference between these compounds was observed in the HMBC correlations of **(2)** with the presence of a connectivity signal between H-3' and C-1''''', corresponding to a 3-methylbutanoyl substituent. The quasimolecular ion peak *m/z* 743 $[M+Na]^+$ obtained by FABMS, as well as fragments at *m/z* 155 (decanoyl), *m/z* 71

(isobutanoyl), and *m/z* 85 (3-methylbutanoyl), supported the structural characteristics of the acyl groups. Peak at *m/z* 247 also corroborates the difference on the furanose moiety substitution and confirms the structure of the new sucrose ester derivative 2-*O*-decanoyl-3,4-di-*O*-isobutyryl-3′-*O*-(3-methylbutanoyl)sucrose, named Peruviose B, $C_{35}H_{60}O_{15}$. After acetylation of this compound, the ion peak at *m/z* 911 $[M+Na]^+$ (calcd. for $C_{43}H_{68}O_{19}Na$) also confirmed the presence of the four original ester groups on the sugar moiety.

Table 3.2. NMR spectroscopic data for Peruviose B (2) in $CDCl_3$ (500 MHz for ^1H and 125 MHz for ^{13}C)

Number	^1H [δ(ppm), *m*, ^3J (Hz)]	^{13}C (δ, ppm)/DEPT	NOESY	HMBC
Glucose-1	5.60, *d, 3.8*	89.42/CH	H-2, H-1'a, H-1'b	C-5, C-2'
2	4.96-4.90 *	70.13/CH	H-1'a	C-3, C-1''
3	5.46, *dd, 10.3, 9.4*	69.05/CH	H-5	C-2, C-4, C-1'''
4	4.90-4.96 *	68.30/CH	H-6	C-3, C-5, C-6, C-1''''
5	4.12-4.19 *	72.01/CH	H-6	
6	3.60, *m*	61.55/CH_2	H-4'	
Fructose-1'a	3.58, *d, 12.0*	64.54/CH_2	H-3', H-5'	
1'b	3.45, *d, 12.0*		H-3'	
2'	—	103.89/C		
3'	5.23, *d, 8.2*	79.12/CH	H-5'	C-1', C-4', C-1'''''
4'	4.56, *t, 8.2*	71.15/CH	H-6'a, H-6'b	C-3', C-5', C-6'
5'	3.96, *m*	82.55/CH		C-4'
6'a	3.92, *m*	59.79/CH_2		C-4'
6'b	3.71, *m*			
1''	—	172,88/C=O		
2''	2.25, *m*	33.85/CH_2	H-3'', H-4''	C-3''
3''	1.54, *m*	24.55/CH_2	H-4''	C-2''
4''-7''	1.24, *s* wide	29.33-29.06/CH_2		
8''	1.24, *s* wide	31.82/CH_2		
9''	1.24, *s* wide	22.63/CH_2		
10''	0.87, *t*, *7.0*	14.06/CH_3		C-8'', C-9''
1'''	—	175.64/C=O		
2'''	2.45, *m*, *7.0*	33.88/CH	H-3'''a, H-3'''b	
3'''a	1.08, *d*, *7.0*	18.79-18.70/CH_3		C-2'''
3'''b	1.07, *d*, *7.0*			C-2'''
1''''	—	176.06/C=O		
2''''	2.52, *m*, *7.0*	33.91/CH	H-3''''a, H-3''''b	C-3''''
3''''a	1.144, *d*, *7.0*	18.79-18.70/CH_3		C-2''''
3''''b	1.119, *d*, *7.0*			C-2''''
1'''''	—	174.06/C=O		
2'''''	2.39, ABX-system, *7.0, 14.8*	43.14/CH_2	H-4'''''a, H-4'''''b	C-4'''''a
3'''''	2.20, *m*	25.86/CH	H-4'''''a, H-4'''''b	C-2'''''
4'''''a	1.05, *d*, *6.7*	22.42/CH_3		C-2''''', C-3''''', C4'''''b
4'''''b	1.04, *d*, *6.7*	22.29/CH_3		C-2''''', C-3''''', C-4'''''a

* The multiplicity could not be determined.

Determination of the absolute configuration of the sugar moiety was performed by alkaline hydrolysis and comparison with a sucrose authentic standard. The product of the hydrolysis of peruviose A and B (**1** and **2**) with NH_4OH (2M) yielded a product with identical absolute configuration ($[\alpha]_D^{20°C}$ product = +62.2) in respect to sucrose standard $[\alpha]_D^{20°C}$ standard = +66).

Acylsucroses are considered the main protective constituents of the resin covering the inner parts of the calyces of several *Physalis* species, since other known sucrose esters exhibit aphicidal, molluscidal, and antifeedant activities.[29] Consequently, we performed an acute toxicity evaluation of the mixture of peruviose A and B, which did not produce mortality or visible signs of toxicity within 24 h when administered intraperitoneally (i.p.) at doses below to 100 mg/Kg. Doses higher than 200 mg/Kg produced toxic effects in a dose dependent-manner with slight hypoactivity, weakness, and labored breathing before animal death, accompanied by permanent piloerection (Table 3.3). The LD_{50} was estimated to be 223.59 (95% confidence interval 209.91-234.52) mg/kg.

Table 3.3. Acute toxicity in mice after 24 h of administration of a mixture of Peruviose A and B isolated from *Physalis peruviana* calyces.

Dose (mg/Kg)[a]	D/T[b]	Mortality Latency[c]	Signs of Toxicity observed
0	0/6	-----	No toxic changes observed
100	0/6	-----	No toxic changes observed. Abdominal contractions.[d]
150	0/6	-----	Abdominal contractions. Slight hypoactivity in the first 30 min.
175	0/6	-----	
200	0/6	-----	Abdominal contractions. Slight hypoactivity in the first 2 h.
215	3/6	>5 h, <24 h	Abdominal contractions. Piloerection. Slight hypoactivity in the first 2 h. Marked hypoactivity, peripheral cyanosis and respiratory arrest before death.
230	4/6	>5 h, <24 h	Abdominal contractions. Piloerection. Marked hypoactivity. Peripheral cyanosis and respiratory arrest before death.
240	5/6	>3 h, <24 h	
250	5/6	>5 h, <24 h	
300	6/6	3-10 h	

a.) The mixture of peruviose A and B was co-precipitated with PVP K-25, dissolved in saline and administered as a single i.p. dose to groups of six mice, which were carefully examined for any signs of behavioral changes and mortality for 24 h. b.) D/T refers to number of mice deaths/total number of mice; c.) Mortality Latency refers to the time to death (in hours) after the injection. d.) Abdominal contractions were noted only after the administration of treatments (10-20 min).

The toxicity of peruviose A and B was significantly high as expected, not only because of the known toxicity of sugar esters [36], but also because of the nearly complete access of tested compounds to general circulation. However, sub-chronic and chronic toxicological evaluations, employing several routes of administration, are needed before further studies with these molecules.

During necropsy, macroscopic examination did not show detectable changes in the shape, color, and size of liver and kidney due to the administration of peruviose A and B. In addition, histopathological evaluation did not reveal significant changes in tissue architecture, inflammatory cell infiltration, swelling or necrosis, in comparison to control group (Figure 3.2). Our results directly demonstrated that toxicity induced by peruviose A and B is not mediated through effects on liver or kidney function. Future studies should include examination of other vital organs like brain, heart, lung, and spleen.

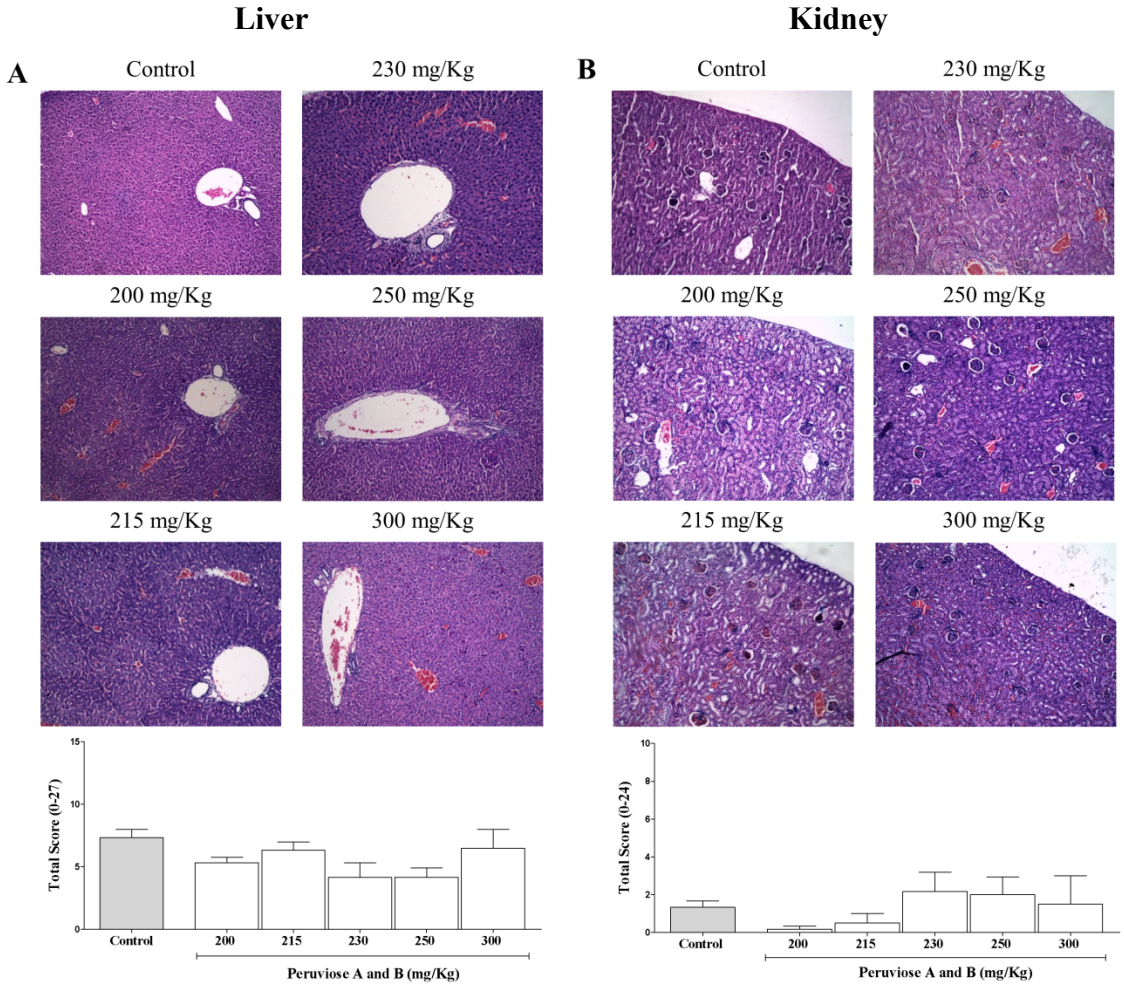


Figure 3.2. Peruviose A and B isolated from *Physalis peruviana* calyces did not produced an effect on the histological structure of liver (A) and kidney (B) of mice treated i.p. with doses of the mixture of sucrose esters ranging from 0 (control) to 300 mg/Kg for 24 h. Micrographs are representative of histological section of organs stained with hematoxylin and eosin (Magnification 10X). A blinded pathologist assigned a score according to the parameters described in Appendix 4. Each value represents the mean±S.E.M (*n*=6).

Based on the experimental LD_{50}, it was decided to select lower doses than 200 mg/Kg to evaluate the anti-inflammatory effect of sucrose esters employing the λ-carrageenan paw-edema test, a classical model of acute inflammation for the discovery of anti-edematous

agents.[37] Sub-plantar injection of λ-carrageenan generated an increase in paw volume of rats in control group, which intensify progressively to reach a maximum peak at 5 h (134.15% of increase). As can be seen in Figure 3.3, peruviose A and B mixture (25, 50, and 100 mg/Kg, i.p.) significantly inhibited the edema induced by λ-carrageenan in a dose-dependent manner as early as 1 h after induction of inflammation. The highest effect of the mixture was produced at 3 h by the dose of 100 mg/kg (62.58±3.35% of inhibition).

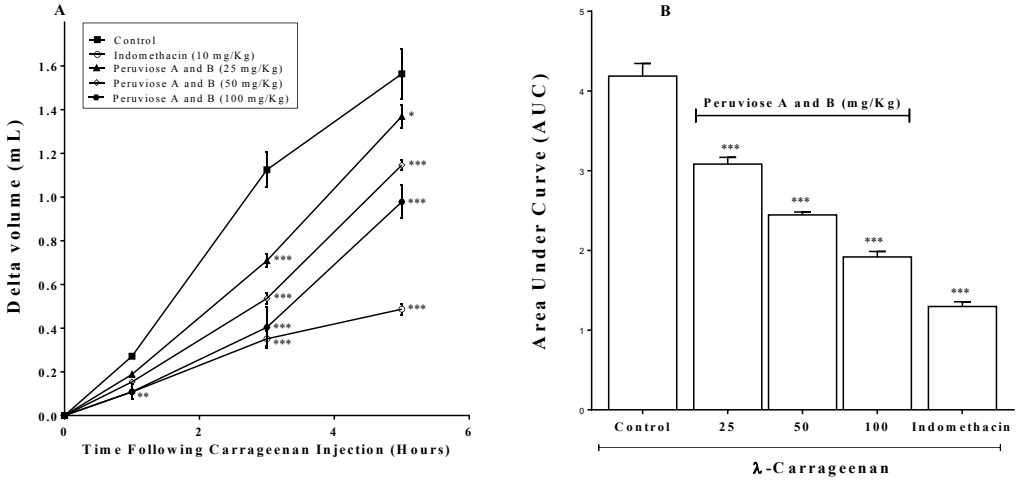


Figure 3.3. Anti-inflammatory effect of the mixture of Peruviose A and B on λ-carrageenan induced paw edema. Six rats per group were administered with sucrose esters (25, 50 and 100 mg/kg, i.p.) or Indomethacin (10 mg/kg, i.p.), 1 h before λ-carrageenan injection. Paw volume was measured at 1, 3 and 5 h intervals, after irritant agent administration, and data expressed as: (A) delta volume (mL), which denotes the degree of swelling after λ-carrageenan treatment or (B) area under curve (arbitrary units). Each value represents the mean±S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001, ANOVA followed by Dunett test, statistically significant compared with control group.

In vivo anti-inflammatory activity of four related sucrose esters isolated from *Physalis sordida* Fernald (CI_{50}=0.24-0.35 μmol/ear), has been recently described.[38] We have also demonstrated the effect of the major glycosidic fraction from *P. peruviana* calyces, which includes peruviose A and B among its constituents, using the TPA-induced ear edema model.[20] These experimental evidences suggest that the presence of sucrose esters can be highly related to the anti-inflammatory activity exerted by some plants of this genus.[38] However, the mechanism underlying this pharmacological activity has not been described.

Edema is an essential feature of acute inflammation caused by increased vascular permeability.[39, 40] The effect induced by λ-carrageenan is a biphasic response with multiple mediators acting in sequence. The first phase (0-1 h) is triggered through the rapid release of histamine, serotonin, and bradykinin. The more pronounced second phase (1-6 h), is elicited by the production of prostaglandins and NO• by inducible isoforms of

cyclooxygenase (COX-2) and nitric oxide synthase (iNOS), respectively.[39, 41] Since our results suggest that the anti-inflammatory effect produced by the mixture of peruviose A and B is due to the inhibition of the synthesis or release of the pro-inflammatory mediators from the second phase, and considering a previous study which reported inhibition of LPS-induced NO• and PGE2 generation on RAW 264.7 macrophages by a supercritical fluid extract of *P. peruviana* leaves [21], we decided to further evaluate the effect of the mixture of sucrose esters from *P. peruviana* calyces, in the production of some of the major mediators of acute inflammation: NO•, PGE2 and TNF-α, by LPS-stimulated mouse peritoneal macrophages.

Macrophages play a pivotal role in host defense against bacterial infection, being the principal cellular target for LPS, the major component in the outer membrane of Gram negative bacteria cell walls, which stimulates the secretion of NO• and PGE2 as well as pro-inflammatory cytokines including TNF-α, interleukin (IL)-1β, and IL-6.[42] To verify whether peruviose A and B are able to inhibit the production of NO• and PGE2, we first evaluate their effect on cell viability, employing MTT assay. As shown in Figure 3.4A, the mixture of Peruvioses inhibited the cell viability in a concentration-dependent manner, LC_{50} 25.41 (15.28-40.24) µg/mL, without exerting significant toxicity at 10 µg/mL. Therefore, concentrations employed in the subsequent experiments were equal or less than this concentration.

NO• is a gaseous signaling molecule that plays a crucial role in host defense mechanisms, via its antimicrobial and cytoprotective activities. Stimulation of murine macrophages by LPS results in the increased expression of iNOS, which catalyzes the production of large amounts of NO•. We found that peritoneal macrophages produced considerable amount of nitrite under basal conditions, 21.82±1.25 µM. However, after stimulation with LPS, nitrite production was increased significantly to a concentration of 65.44±3.42 µM. Non-toxic concentrations of the mixture of peruviose A and B produced a significant reduction of nitrite production dependent on the concentration, IC_{50}=2.317 (1.368-4.055) µg/mL, showing a similar activity for that presented by 1400W (Figure 3.4B), without exerting an important scavenging effect of NO• (Data not shown), indicating that suppression of its release can be directly attributed to a blocked production by stimulated macrophages.

PGE2 is known to be a key mediator of immunopathology in chronic inflammatory diseases and cancer [43]. As it can be seen in Figure 3.4C, LPS produced a significant increase of PGE2 levels in cell culture, changing its concentration from 4.18±0.38 ng/mL to 27.43±0.61 ng/mL. The mixture of peruviose A and B exhibited a potent inhibitory effect on PGE2 release in cell culture in a concentration-dependent manner, with IC_{50}=0.072 (0.019-0.293) µg/mL. The effects of the mixture of sucrose esters are comparable to those of Rofecoxib, which is a selective COX-2 inhibitor.

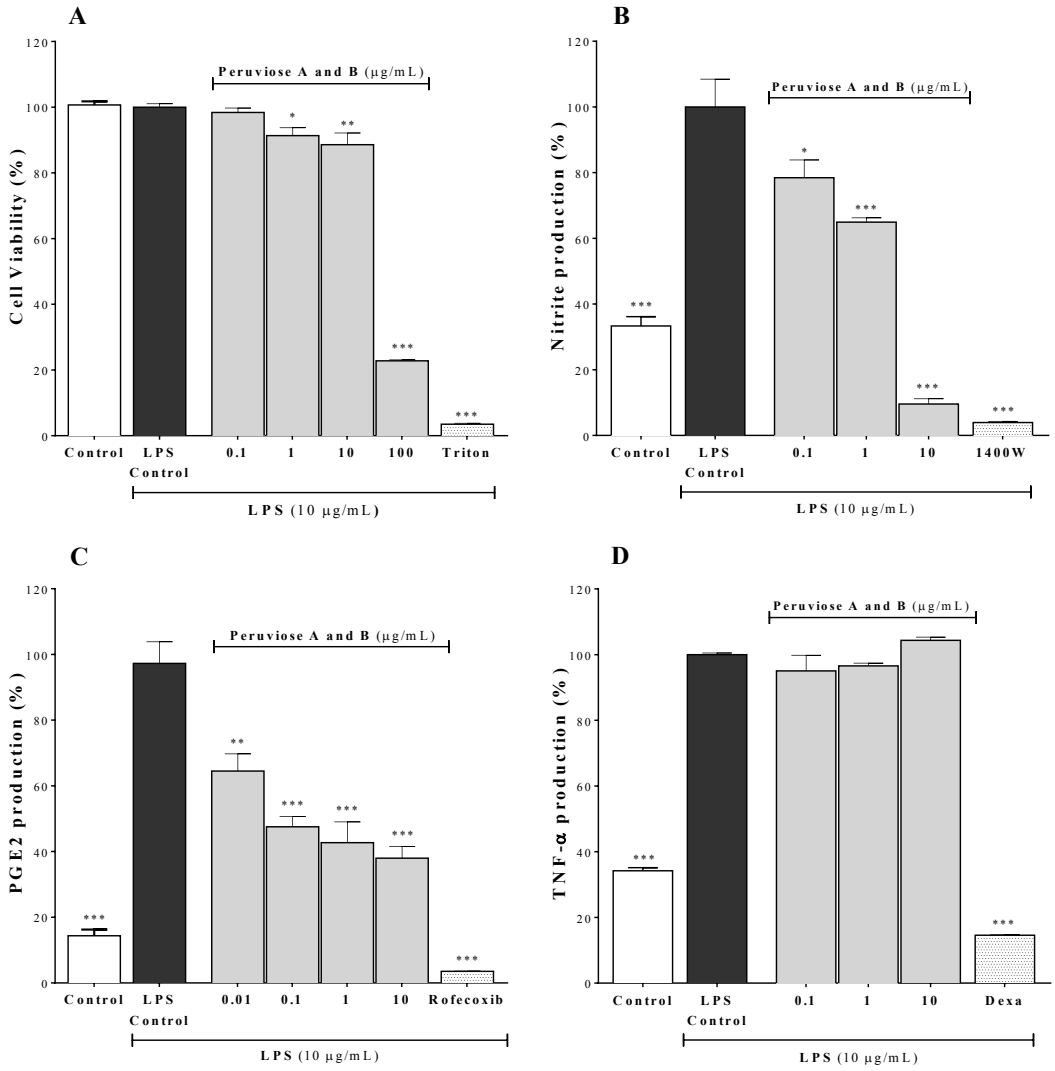


Figure 3.4. Effect of the mixture of peruviose A and B on LPS-induced mouse peritoneal macrophages viability and pro-inflammatory mediators production. Macrophages were treated with various concentrations of the mixture of sucrose esters (0.01-100 µg/mL) for 30 min, and activated with LPS (10 µg/mL) for 24 h. After incubation cell viability was determined by MTT assay (A) or culture supernatants were collected. Nitrite production was assessed using Griess reaction (B) and ELISA was employed to quantify PGE2 (C) and TNF-α release (D). Triton X-100® (20%), 1400W (2.50 µg/mL), Rofecoxib (6.29 µg/mL), and Dexamethasone (7.85 µg/mL) were employed as positive controls, respectively. Results are expressed as the mean±SME of at least two independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, ANOVA followed by Dunett test, compared with LPS treated group.

Interestingly, *In vitro* anti-inflammatory activity through inhibition of PGE2 production, of LPS-induced RAW 264.7 macrophages, by three sucrose esters isolated from *Bidens parviflora* Willd was also described.[44] Taken together, these results suggest that the anti-inflammatory activity of sucrose esters might be related with inhibition of this important prostanoid.

TNF-α is a potent pro-inflammatory cytokine released primarily from stimulated macrophages playing a critical role in the host response to infection and injury.[45] LPS-stimulated macrophages significantly increased TNF-α production in 2.93-fold (Figure 3.4D). Unexpectedly, the mixture of peruviose A and B did not affect the production of TNF- α, even at the highest tested concentration, which suggests that the anti-inflammatory activity of these sucrose esters is mediated through selective downregulation of iNOS and COX-2, independently of common pathways or transcription factors with TNF-α.

It has been suggested that NO• play a key role in the modulation of PGE2 synthesis in macrophages. In fact, a large body of evidence suggests that there is significant cross talk between iNOS and COX-2 biosynthetic pathways, especially in biological systems like LPS-stimulated peritoneal macrophages.[46] However, the final effect of these interactions is often unclear, varying between different kinds of cells and tissues.[47, 48] Further studies are needed to clarify the effect of peruviose A and B on iNOS and COX-2 in peritoneal macrophages.

In conclusion, we described the isolation of a mixture of two new anti-inflammatory sucrose esters from the calyces of *P. peruviana*. Overall, our results suggest that sucrose esters are important anti-inflammatory compounds of *Physalis* genus, mainly through down-regulation of NO• and PGE2 production.

3.5 References

1. Sobolewski, C., et al., *Inflammation: novel arrows for an ancient target.* Biochemical pharmacology, 2010. **80**(12): p. 1769-1770.
2. Wittmann, C., et al., *Hydrogen Peroxide in Inflammation: Messenger, Guide, and Assassin.* Advances in Hematology, 2012. **2012**: p. 1-6.
3. Al-Reza, S.M., et al., *Anti-inflammatory activity of seed essential oil from Zizyphus jujuba.* Food and Chemical Toxicology, 2010. **48**(2): p. 639-643.
4. Burk, D.R., et al., *Suppression of lipopolysaccharide-induced inflammatory responses in RAW 264.7 murine macrophages by aqueous extract of Clinopodium vulgare L. (Lamiaceae).* Journal of ethnopharmacology, 2009. **126**(3): p. 397-405.
5. Morley, J., *Inflammation and asthma therapy: a false dawn.* Pulmonary pharmacology & therapeutics, 2006. **19**(3): p. 200.
6. Vidal-Vanaclocha, F., *Inflammation in the molecular pathogenesis of cancer and atherosclerosis.* Reumatología Clínica, 2009. **5**(S1): p. 40-43.
7. Mustafa, A., et al., *Ginkgo biloba attenuates mucosal damage in a rat model of ulcerative colitis.* Pharmacological research, 2006. **53**(4): p. 324-330.

8. Llorca, J., et al., *Persistent chronic inflammation contributes to the development of cancer in patients with rheumatoid arthritis from a defined population of northwestern Spain.* Seminars in Arthritis and Rheumatism, 2007. **37**(1): p. 31-38.
9. Heo, S.-J., et al., *Evaluation of anti-inflammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stimulated RAW 264.7 macrophages.* Food and Chemical Toxicology, 2010. **48**(8–9): p. 2045-2051.
10. Wu, S.J., et al., *Antihepatoma activity of Physalis angulata and P. peruviana extracts and their effects on apoptosis in human Hep G2 cells.* Life sciences, 2004. **74**(16): p. 2061-2073.
11. Chang, J., et al., *Antioxidative and hepatoprotective effects of Physalis peruviana extract against acetaminophen-induced liver injury in rats.* Pharmaceutical Biology, 2008. **46**(10-11): p. 724-731.
12. Wu, S.J., et al., *Antioxidant activities of Physalis peruviana.* Biological and Pharmaceutical Bulletin, 2005. **28**(6): p. 963-966.
13. Wu, S.J., et al., *Physalis peruviana extract induces apoptosis in human Hep G2 cells through CD95/CD95L system and the mitochondrial signaling transduction pathway.* Cancer letters, 2004. **215**(2): p. 199-208.
14. Yen, C.Y., et al., *4β-Hydroxywithanolide E from Physalis peruviana (golden berry) inhibits growth of human lung cancer cells through DNA damage, apoptosis and G2/M arrest.* BMC cancer, 2010. **10**(1): p. 46.
15. Wu, S.J., et al., *Supercritical carbon dioxide extract of Physalis peruviana induced cell cycle arrest and apoptosis in human lung cancer H661 cells.* Food and Chemical Toxicology, 2009. **47**(6): p. 1132-1138.
16. Lan, Y.H., et al., *New cytotoxic withanolides from Physalis peruviana.* Food Chemistry, 2009. **116**(2): p. 462-469.
17. Arun, M. and V. Asha, *Preliminary studies on antihepatotoxic effect of Physalis peruviana Linn.(Solanaceae) against carbon tetrachloride induced acute liver injury in rats.* Journal of ethnopharmacology, 2007. **111**(1): p. 110-114.
18. Mora, A.C., D.M. Aragón, and L.F. Ospina, *Effects of Physalis peruviana fruit extract on stress oxidative parameters in streptozotocin-diabetic rats.* Latin American Journal of Pharmacy, 2010. **29** (7): p. 1132-1136.
19. Martínez, W., et al., *In vitro studies on the relationship between the anti-inflammatory activity of Physalis peruviana extracts and the phagocytic process.* Immunopharmacology and Immunotoxicology, 2010. **32**(1): p. 63-73.
20. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica: revista del Instituto Nacional de Salud, 2007. **27**(1): p. 110.
21. Wu, S., et al., *Supercritical carbon dioxide extract exhibits enhanced antioxidant and anti-inflammatory activities of Physalis peruviana.* Journal of ethnopharmacology, 2006. **108**(3): p. 407-413.
22. Trinchero, G.D., et al., *Ripening-related changes in ethylene production, respiration rate and cell-wall enzyme activity in goldenberry (Physalis peruviana L.), a solanaceous species.* Postharvest Biology and Technology, 1999. **16**(2): p. 139-145.
23. Puente, L.A., et al., *Physalis peruviana Linnaeus, the multiple properties of a highly functional fruit: A review.* Food Research International, 2011. **44**(7): p. 1733-1740.
24. Valdenegro, M., et al., *Changes in antioxidant capacity during development and ripening of goldenberry (Physalis peruviana L.) fruit and in response to 1-methylcyclopropene treatment.* Postharvest Biology and Technology, 2012. **67**: p. 110-117.
25. Mazorra, M.F., et al., *Anatomic aspects of formation and growth of the cape gooseberry fruit Physalis peruviana (Solanaceae).* Acta Biológica Colombiana, 2006. **11**(1): p. 69-81.

26. Mayorga, H., et al., *Hydroxyester disaccharides from fruits of cape gooseberry (Physalis peruviana).* Phytochemistry, 2002. **59**(4): p. 439-445.
27. Ovenden, S.P.B., et al., *Physaloside A, an acylated sucrose ester from Physalis viscosa.* Journal of Natural Products, 2005. **68**(2): p. 282-284.
28. Pérez-Castorena, A.-L., et al., *New sucrose esters from the fruits of Physalis solanaceus.* Carbohydrate Research, 2012. **352**(0): p. 211-214.
29. Maldonado, E., et al., *Sucrose esters from the fruits of Physalis nicandroides var. attenuata.* Journal of Natural Products, 2006. **69**(10): p. 1511-1513.
30. Godin, P., *A new spray reagent for paper chromatography of polyols and cetoses.* Nature, 1954. **174**(4420): p. 134-134.
31. Miller, L.C., M.L. Tainter, and *Estimation of the ED_{50} and its Error by means of logarithmic-probit graph paper.* Exp Biol Med, 1944. **57**(2): p. 261-264.
32. Winter, C.A., E.A. Risley, and G.W. Nuss, *Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs.* Proc Soc Exp Biol Med, 1962. **111**: p. 544-7.
33. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays.* Journal of Immunological Methods, 1983. **65**(1–2): p. 55-63.
34. Ferrari, M., M.C. Fornasiero, and A.M. Isetta, *MTT colorimetric assay for testing macrophage cytotoxic activity in vitro.* Journal of Immunological Methods, 1990. **131**(2): p. 165-172.
35. Green, L.C., et al., *Analysis of nitrate, nitrite, and (^{15}N)nitrate in biological fluids.* Analytical Biochemistry, 1982. **126**(1): p. 131-138.
36. Gruda, I., et al., *Structure-activity study of inhibition of amphotericin B (Fungizone) binding to sterols, toxicity to cells, and lethality to mice by esters of sucrose.* Antimicrobial Agents and Chemotherapy, 1991. **35**(1): p. 24-28.
37. Buapool, D., et al., *Molecular mechanism of anti-inflammatory activity of Pluchea indica leaves in macrophages RAW 264.7 and its action in animal models ofinflammation.* J Ethnopharmacol, 2013. **146**(2): p. 495-504.
38. Perez-Castorena, A.L., M. Martinez, and E. Maldonado, *Labdanes and sucrose esters from Physalis sordida.* J Nat Prod, 2010. **73**(7): p. 1271-6.
39. Ferreira, L.C., et al., *Anti-inflammatory and antinociceptive activities of Campomanesia adamantium.* J Ethnopharmacol, 2013. **145**(1): p. 100-8.
40. Zanoni, I., et al., *CD14 and NFAT mediate lipopolysaccharide-induced skin edema formation in mice.* J Clin Invest, 2012. **122**(5): p. 1747-57.
41. Vinegar, R., W. Schreiber, and R. Hugo, *Biphasic development of carrageenin edema in rats.* J Pharmacol Exp Ther, 1969. **166**(1): p. 96-103.
42. Moita, E., et al., *Integrated analysis of COX-2 and iNOS derived inflammatory mediators in LPS-stimulated RAW macrophages pre-exposed to Echium plantagineum L. bee pollen extract.* PLoS One, 2013. **8**(3): p. e59131.
43. Kalinski, P., *Regulation of immune responses by prostaglandin E2.* J Immunol, 2012. **188**(1): p. 21-8.
44. Wang, N., et al., *Bioactive sucrose esters from Bidens parviflora.* Phytochemistry, 2003. **62**(5): p. 741-6.
45. Niu, X., et al., *Isofraxidin exhibited anti-inflammatory effects in vivo and inhibited TNF-alpha production in LPS-induced mouse peritoneal macrophages in vitro via the MAPK pathway.* Int Immunopharmacol, 2012. **14**(2): p. 164-71.
46. Medeiros, A., et al., *Prostaglandin E2 and the suppression of phagocyte innate immune responses in different organs.* Mediators Inflamm, 2012. **2012**: p. 327568.
47. Mollace, V., et al., *Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors.* Pharmacol Rev, 2005. **57**(2): p. 217-52.

48. Salvemini, D., et al., *Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors.* J Clin Invest, 1995. **96**(1): p. 301-8.

4 CHAPTER FOUR. Cape gooseberry (*Physalis peruviana* L.) calyces ameliorate TNBS-induced colitis in rats

Jenny Castro[1*]. Yanet Ocampo[1*], Luis Franco[1].

[1]Biological Evaluation of Promising Substances Group. Faculty of Pharmaceutical Sciences. University of Cartagena. Cartagena, Colombia.
*Both authors contributed equally to this work as first authors.

4.1 Abstract

Physalis peruviana (Cape-Gooseberry) is highly appreciated for its commercial value; the ecotype Colombia has a great demand on the international markets and unique morphological characteristic in the calyx, which has extended use as a traditional herbal remedy in Colombia for its anti-inflammatory properties. In this work, we evaluated the anti-inflammatory activity of the total ethereal extract of *P. peruviana* calyces in preventive and therapeutic protocols in a TNBS acid-induced colitis rat model. Colitis was induced by intrarectal administration of TNBS. An evaluation of macroscopic and histological parameters in colonic tissue was performed; along with the determination of myeloperoxidase enzyme activity, gene expression, and cytokine levels. Additionally, effects on NO release by LPS-stimulated RAW 264.7 macrophages and the scavenging activity of DPPH and ABTS free radicals were determined. The treatment with the *P. peruviana* extract produced a significant improvement in the colonic tissue at both macroscopic and histological levels. IL-1β and TNF-α production was reduced by the extract in both experimental approaches. The groups treated with *P. peruviana* showed a tendency to MUC2 up-regulation and down-regulation of COX-2, iNOS, NLRP3, IL-1β, IL-6 and IL-10 expression. NO release in RAW 264.7 macrophages was significantly inhibited. The *P. peruviana* extract showed intestinal anti-inflammatory activity in the TNBS-induced colitis model, placing this species´ calyx, a natural derivative, as a promising source of metabolites that could be used as a natural product derived treatment for inflammatory bowel disease.

Keywords: *Physalis peruviana*, Inflammatory Bowel Disease, TNBS.

4.2 Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that comprises Crohn's disease, ulcerative colitis, and indeterminate colitis.[1, 2] Although IBD etiology is still not clear, it is known that its appearance implies the interaction of genetic and environmental factors, along with alterations on the immune response of the host.[3, 4] It is characterized by the infiltration of lymphocytes, macrophages, neutrophils, and dendritic cells capable of destabilize the integrity of the

mucosa and generate histologic lesions through the synthesis and liberation of a variety of pro-inflammatory mediators that include reactive oxygen species, eicosanoids, platelets activation factor, nitrogen metabolites, and cytokines, which actively contribute to the pathogenic cascade that starts and maintains the chronic inflammatory response in the gut.[5, 6] There is no cure for this pathology, partially because the triggering factors have not been properly identified, thus the therapeutic strategies have aimed to interfere on different stages of the inflammatory process and regulate the overactive immune system. Nevertheless, these therapeutic approaches are limited by their low efficacy and multiple secondary effects.[7] Therefore, the development of safer and more effective therapeutic alternatives to treat this disease has become a priority. In this sense, plants may be a promising source of new therapeutic agents for the treatment of IBD. Several studies report plant extracts with anti-inflammatory activity exerted through the modulation of various inflammatory mediators including IL-1β, IL-6, IL-10, TNF-α, nitric oxide (NO), and prostaglandin E2 (PGE-2). Some of these extracts are clinically used due to their beneficial effects.[8, 9]

Physalis peruviana L., (Solanaceae) native from the South American Andes, is one of the most known species of this genus. This plant grows in areas about 2200 m above sea levels, and is currently found in almost every tropical and some subtropical meadow including Malaysia, China, and the Caribbean.[10, 11] Most of the organs from this species are widely employed in folk medicine due to their diuretic, antiseptic, antifungal, antibacterial, anti-carcinogenic, antimalarial, and anti-inflammatory effects.[10-13] Regarding the study of the calyces of *P. peruviana*, there are only a few reports of its anti-inflammatory activity;[14-17] and to our knowledge, this is the first work in which this organ is studied using an animal model of IBD. In this study, the protective and curative effect of the total ethereal extract of the calyces of *P. peruviana* L. in a TNBS-induced colitis model in rats was evaluated. Colonic tissue obtained from the animals was submitted to macroscopic and histologic analysis. Myeloperoxidase (MPO) enzyme activity was determined, as well as the levels of TNF-α, INF-γ, IL-1β, IL-4, IL-6, and IL-10; and the differential expression of the COX-2, iNOS, MUC2, NLRP3, IL-1β, IL-6, IL-10 and IL-17 genes. Complementarily, the effect of the extract of *P. peruviana* on the NO production was evaluated *in vitro* on RAW 264.7 macrophages and its scavenging activity was measured using DPPH and ABTS assays.

4.3 Materials and Methods

4.3.1 Reagents

Dulbecco´s Modified Eagle Medium (DMEM), Penicillin-Streptomycin, trypan blue, lipopolysaccharide from *E. coli* (LPS), N-([3(aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride (1400W), sodium nitrite, N-(1,1-naphthyl) ethylenediamine

dihydrochloride, sulfanilamide, hematoxylin, eosin, trinitrobenzenesulfonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$) were purchased from Sigma Aldrich (St. Louis, MO, USA). Bromide of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were obtained from Calbiochem® (San Diego, CA, USA). Polyvinylpyrrolidone K-25 (PVP K-25) was USP grade. Petroleum ether, chloroform and ethanol were acquired from Merck. Fetal bovine serum (FBS) from GIBCO (Gaithersburg, MD, USA), dimethylsulfoxide from Carlo Erba, diazepam from Carlon S.A. (Bogotá, Colombia), and ketamine from Lab Biosano (Santiago de Chile, Chile). Macrophages RAW 264.7 were acquired from the American Type Culture Collection, ATCC (Manassas, VA, USA).

4.3.2 Animals

Female Wistar rats and ICR mice (6–8 weeks of age) were obtained from the Instituto Nacional de Salud de Colombia. They were kept in a stress-free and controlled environment, at 22±3 ºC and 70% ± 5% relative humidity, under 12-h cycles of light and darkness. Food and water were provided *ad libitum*. All experiments were designed and conducted in accordance with the guidelines of the Ethics Committee of the University of Cartagena (Minutes of October 14 of 2010) and the European Union regulations (CEC council 86/809).

4.3.3 Extract Preparation

Physalis peruviana calyces were collected at La Mesa, Cundinamarca, and a voucher specimen was sent to the Herbario Nacional Colombiano of the National University of Colombia for its identification and was filed under the code COL512200. Plant material (2 Kg) was dried in an airflow oven at a constant temperature of 40 ºC. Subsequently; it was grounded and extracted by percolation with petroleum ether until exhaustion and concentrated on a rotary evaporator with reduced pressure and controlled temperature (35-45 ºC) to obtain 200 g of total extract. Given the low solubility of *P. peruviana* extract in aqueous solution, it was necessary to co-precipitate it with PVP K-25 in a extract:PVP (1:4 w/w) ratio and was solubilized in PBS buffer for administration. Qualitative identification of alkaloids, flavonoids, nafto and/or anthraquinones, tannins, saponins, terpenoids, coumarins, terpene and lactones was performed by the protocols established by Sanabria-Galindo *et al.* 1997 [18] and the identification of glycosides according to the protocol of Tiwari, Kumar *et al.* 2011.[19]

4.3.4 Acute toxicity

To define the adequate doses for *in vivo* anti-inflammatory activity assays, the acute toxicity of the *P. peruviana* extract was evaluated for a 24 hours period. Lethal dose 50 (LD_{50}) was determinate following the protocol described by Al-Sultan, *et al 2006,* with some modifications.[20] Briefly, ICR mice were randomly distributed in seven groups of

six animals and administrated intraperitoneally (i.p.) with doses between 300-450 mg/kg of *P. peruviana* extract, selected according to previous studies. For histological analysis, liver and kidney samples were preserved in buffered formalin and stained with hematoxylin and eosin.

4.3.5 Induction of colitis and treatments

Colitis was induced employing the method described by Morris, *et al 1989*, with some modifications.[21] Briefly, Wistar rats were not fed for 12 h and anesthetized with a mixture of ketamine (100 mg/Kg) and diazepam (5 mg/Kg) i.p. Subsequently, 0.25 mL of an ethanolic solution of TNBS (40 mg/mL) was instilled rectally using a cannula that was introduced to an 8 cm depth. A healthy control group was included, which was instilled with saline solution.

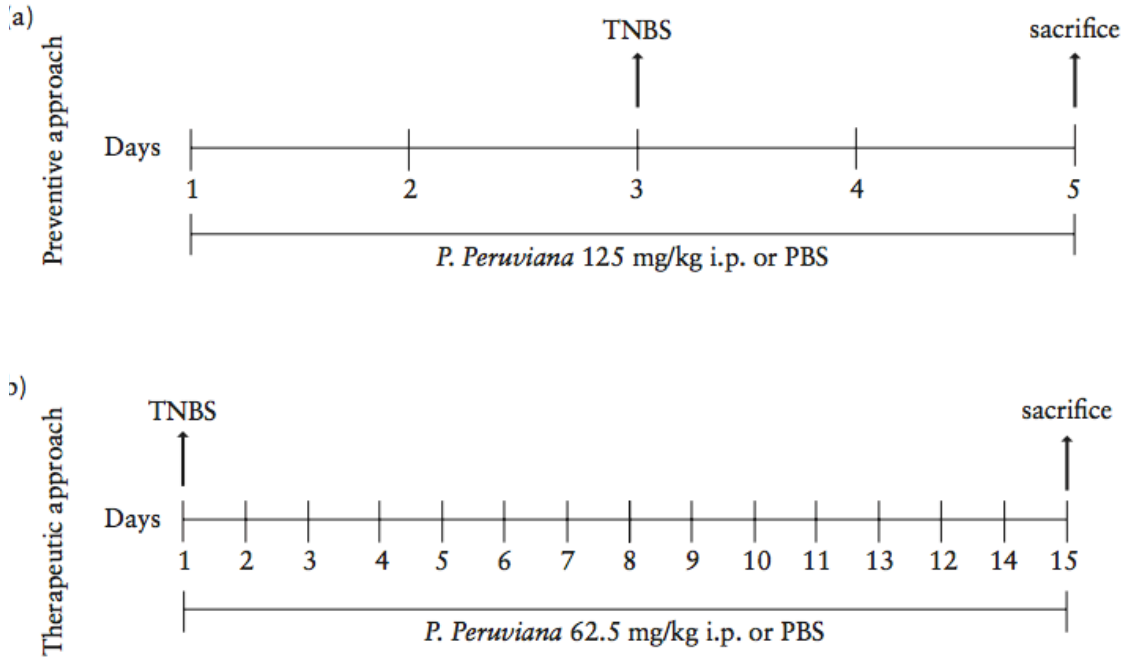


Figure 4.1. Experimental design of TNBS- acid induced colitis. Wistar rats (*n*=10 per group) were rectally instilled with 0.25 mL of TNBS ethanolic solution (40 mg/mL) and with *Physalis peruviana* extract (125 mg/Kg/day-preventive approach or 62.5 mg/Kg/day-therapeutic approach) or phosphate buffer saline (PBS) intraperitoneally (ip). In both assays a Sham group was included.

The different doses of the *P. peruviana* extract used were calculated as about 1/3 and 1/6 of its LD_{50}. The anti-inflammatory effect of the extract was studied using two different protocols (Figure 4.1). In the first approach the protective effect was evaluated, rats received 125 mg/kg i.p., at 48, 24 and 2h before colitis induction as well as 24 h thereafter. In the second outline the therapeutic effect was evaluated administrating a dose of 62.5 mg/kg i.p., for 15 days and the colitis induction was performed from the first day of treatment. The healthy control and TNBS groups were treated with sterile PBS as a vehicle.

Animal weight and food consumption was monitored on a daily basis. At the end of each experiment the animals were sacrificed by cervical dislocation, the colon was extracted, cleaned, weighted and macroscopically analyzed, determining a colonic tissue damage score using the scale of Bobin-Dubigeon *et. al.,* 2001.[22] A colonic tissue sample was preserved in buffered formalin, embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin or Periodic Acid-Schiff (PAS) for histological analysis. The rest of the colon was kept for subsequent determination of the MPO activity, cytokine levels, and gene expression analysis through RT-PCR.

4.3.6 MPO activity on colonic tissue

The enzyme activity was measured according to the technique described by Castro *et al.* 2014*,*[23] and the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 mmol hydrogen peroxide/min at 25 °C.[24]

4.3.7 Cytokine levels on colonic tissue

The colonic tissue was homogenized in Greenburger buffer supplemented with proteases inhibitors (cOmplete Mini EDTA free Roche), sonicated for 10 seconds and centrifuged at 10000 rpm and 4 ºC for 20 minutes.[25] The levels of TNF-α, INF-γ, IL-1β, IL-4, IL-6, and IL-10 were quantified on the obtained supernatants using commercial ELISA kits (Invitrogen, Carlsbad, CA, USA and R&D Systems, Minneapolis, MN, USA for IL-1β), according to the manufacturer's protocol. The results of cytokine release were normalized by the protein content, which was quantified by the Bradford method, using a standard commercial kit (Biorad 500-0206).[26]

4.3.8 Gene expression on colonic tissue by RT-PCR

Extraction of total mRNA from the tissue obtained in the therapeutic experiment was performed with a commercial kit (QIAGEN RNeasy® 74106), following the instructions provided by the manufacturer and quantified in a Nanodrop 2000c (Thermo Scientific). The cDNA was synthesized using QIAGEN Quantitect® 205313 kit and amplified with power SYBR® Green PCR master mix (Applied Biosystems) and specific primers – Appendix 5 (Eurofins Genomics) in a RT-PCR LightCycler® 96 System (Roche). The mRNA relative expression of COX-2, iNOS, MUC2, NLRP3, IL-1β, IL-6, IL-10 and IL-17 was calculated using the ΔΔCt method and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

4.3.9 NO production by RAW 264.7 macrophages

RAW 264.7 macrophages were maintained on DMEM supplemented with 10% FBS and penicillin-streptomycin; at 37ºC in a 5% CO2 and 100% humidity atmosphere.[27] The cell viability was evaluated employing MTT colorimetric assay.[28] To determine the effect of *P. peruviana* extract on NO release; RAW 264.7 macrophages (2x105 cells/mL) were incubated on 96 well plates with non-toxic concentrations of the sample or 1400W. After

30 minutes, cells were stimulated with LPS (1 µg/mL) and incubated again for 24h in the previously described conditions. The Griess reagent was added to the obtained supernatants in a 50/50 ratio and the OD_{550} was determined in a Multiskan EX Thermo microplate reader. Nitrite concentration on the supernatants was established using a standard curve of $NaNO_2$. In each assay was included a non-stimulated control group and a group treated with just LPS.[29] Additionally, the NO scavenging effect of the extract was evaluated using the method previously described by Castro, *et al* 2014.[23]

4.3.10 Antioxidant activity

The scavenging activity of the extract of *P. peruviana* on the DPPH and ABTS radicals was determined spectrophotometrically, employing protocols adapted for microplate reading. For the DPPH, 75 µL of the extract were added to 150 µL of a DPPH solution (100 µg/mL), this mixture was incubated at 25ºC for 30 minutes and the OD_{550} was measured.[30] To determine the effect on the ABTS radical, 10 µL of the extract were mixed with 190 µL of an ABTS ethanolic solution, produced by the reaction of ABTS (3,5 mM) with potassium persulfate (1.25 mM), incubated in the dark for 16h at room temperature and diluted with ethanol to an absorbance of 0.7±0.1 at 405 nm. The final mixture was incubated at 25ºC for 5 minutes and the OD405 was determined.[31] In both assays vehicle and Trolox were used as negative and positive controls, respectively. The results are shown as µmol Trolox/g of calyces.

4.3.11 Statistical Analysis

The results are expressed as the mean ± SEM and analyzed employing one-way ANOVA, followed by Tukey *post hoc*. Values of $P<0.05$ were considered significant. The LD_{50} was calculated using the Probit method.

4.4 Results

4.4.1 *Physalis peruviana* Extract

The preliminary phytochemical analysis of the total ethereal extract from calyces of *P. peruviana,* allowed the identification of the presence of flavonoids, terpenoids, and glycosides. With regard to its toxicity, the estimated DL_{50} was 345.49 mg/Kg i.p. (confidence interval 311.03-379.95 mg/Kg). The histological analysis showed that *Physalis peruviana* extract, at doses lower than 450 mg/Kg, produced no damage to the liver and kidney (data not shown).

4.4.2 *Physalis peruviana* anti-inflammatory effect on rats with colitis.

The administration of TNBS significantly decreased food consumption and a consequent weight lost was observed, just as in human clinical pathology. In both approaches, the animals of the TNBS and *P. peruviana* groups suffered a drastic weight loss compared with

the healthy control group and significant differences were not observed between TNBS and *P. peruviana* groups (Figure 4.2).

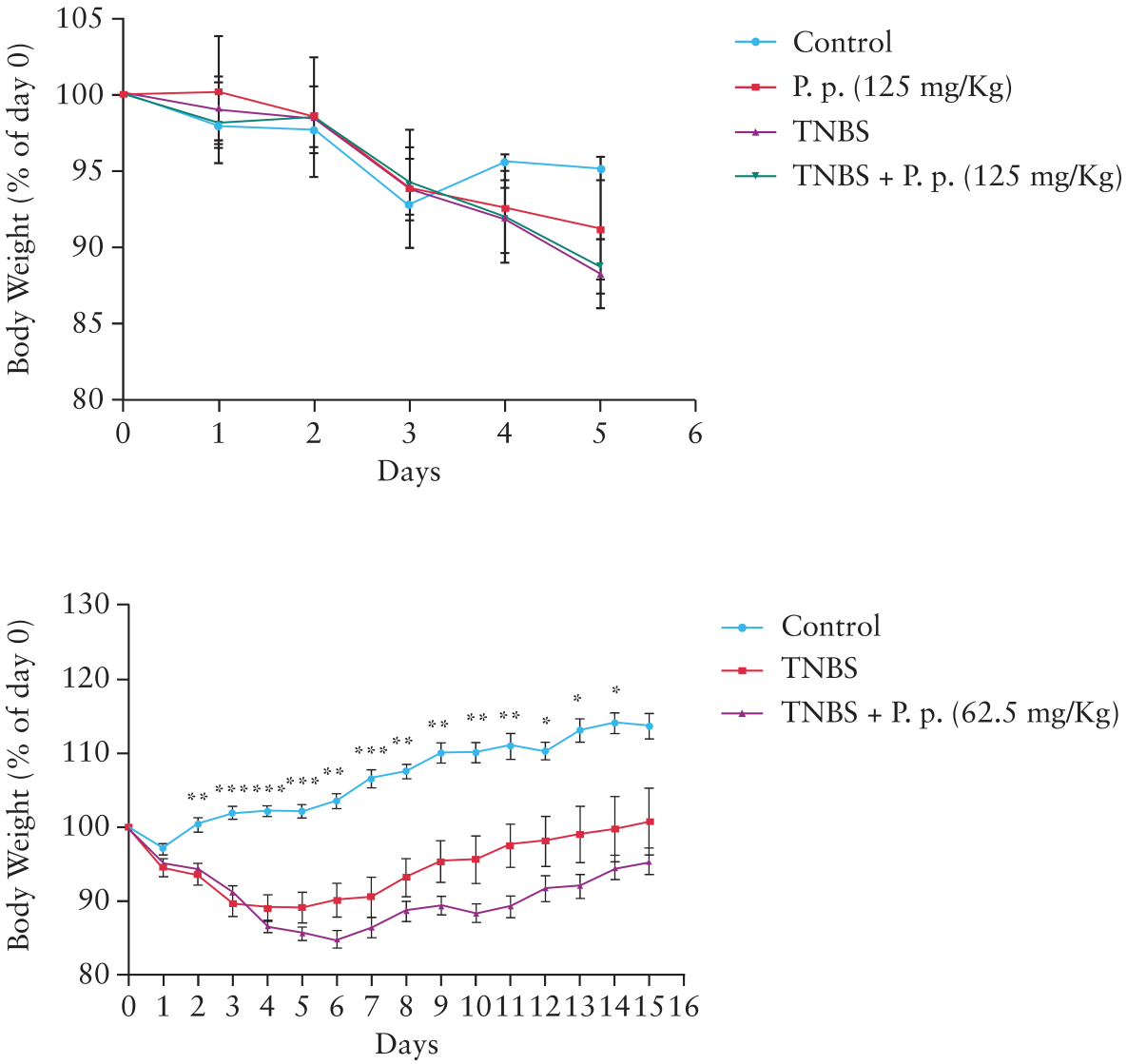


Figure 4.2. Body weight changes following the TNBS colitis induction. A: Preventive approach (*Physalis peruviana* (P. p) extract 125 mg/kg/day i.p.) B: Therapeutic approach (*Physalis peruviana* (P. p) extract 62.5 mg/kg/day i.p.). The results represent the daily changes in body weight in Wistar rats per group. Body weight change was calculated by dividing body weight on the specified day by body weight at day 0 (starting body weight) and expressed in percentage ± SEM. (n=10). (*P<0.05; **P<0.01; ***P<0.001 ANOVA, statistically significant against TNBS).

In both experimental approaches, TNBS produced damage of the intestinal mucosa of the rats, with transmural involvement, ulcerations, edema, and tissue thickening (Figure 4.3B and 4.3E), while the healthy groups kept their normal structure (Figure 4.3A and 4.3D). The treatment with the extract of *P. peruviana* (Figure 4.3C and 4.3F) produced a

significant improvement on the colonic tissue, decreasing the damage score, extension of damaged areas, and weight/length ratio of the tissue (Table 4.1).

Table 4.1. Effect of *Physalis peruviana* (*P.p*) extract on the damage score, damaged area and the weight/length ratio, in the TNBS-induced colitis.

Experiment	Group	Damage score (0-10)	Damaged area (mm^2)	Weight/Length ratio (mg/cm)
Preventive: 5 days	Sham	0.0***	0.0***	69.4 ± 4.2***
	TNBS	8.1 ± 0.6	627.4 ± 69.5	133.7 ± 10.1
	P.p	0.0***	0.0***	76.7 ± 5.3***
	TNBS + *P.p*	3.4 ± 0.6***	305.3 ± 76.7***	89.4 ± 4.5**
Therapy: 15 days	Sham	0.0***	0.0***	62.62 ± 2.1***
	TNBS	8.4 ± 0.9	1357.7 ± 340.0	450.6 ± 101.87
	TNBS + *P.p*	4.4 ± 0.4**	630.02 ± 41.35*	192.0 ± 9.6*

Sham and TNBS groups were treated with PBS in both approaches. *P.p* was administered at 125 mg/kg/day i.p. (Preventive approach) and 62.5 mg/kg/day i.p. (Therapeutic approach). Values represent the mean ± SEM (n = 10). (**P*<0.05; ***P*<0.01; ****P*<0.001 ANOVA, statistically significant against TNBS).

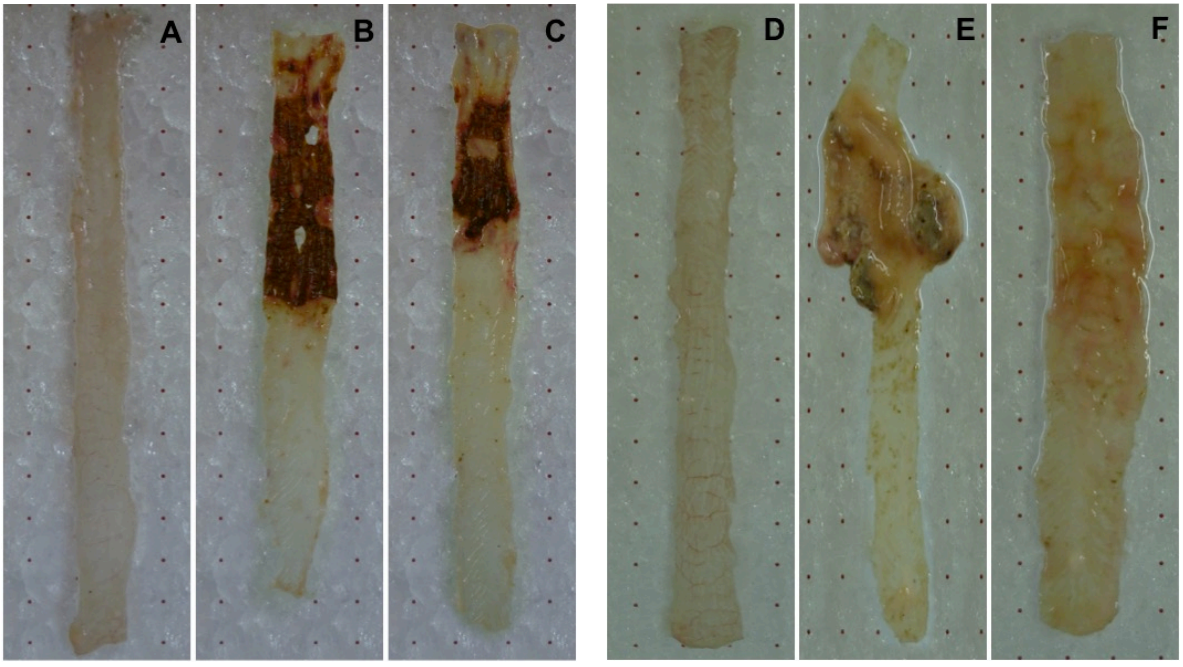


Figure 4.3. Colonic segments of the groups included in the colitis experiment. Preventive approach: (A) Sham, (B) TNBS acid and (C) TNBS + *Physalis peruviana* extract (125 mg/kg/day i.p.). Therapeutic approach: (D) Sham, (E) TNBS and (F) TNBS + *Physalis peruviana* extract (62.5 mg/kg/day i.p.).

Histologically, the animals from the healthy control groups showed typical characteristics of a normal structure (Figure 4.4A and 4.4E), while the TNBS group showed an intense transmural interruption, extensive ulceration, inflammation, edema and massive infiltration of neutrophils mainly in the mucosa (Figure 4.4B and 4.4F). In the preventive approach, the animals treated with *P. peruviana* extract, showed less edema and neutrophil infiltration, as well as a restoration of the mucosa, submucosa and muscular layers (Figure 4.4C and 4.4D). The therapeutic approach, on the other hand, showed restoration limited to the mucosa (Figure 4.4G and 4.4H), in contrast, with the TNBS group that presented crypt loss and goblet cells depletion (Figure 4.5).

4.4.3 MPO activity

MPO enzyme is mainly produced by neutrophils. The results of the *P. peruviana* extract evaluation showed a slight reduction of MPO activity induced by TNBS in both approaches (Figure 4.6), suggesting a decreased infiltration of neutrophils in the colonic tissue, which is consistent with the observed in the histological analysis.

4.4.4 Cytokine levels

TNBS administration produced a reduction of IL-4 levels and an increase of TNF-α, IFN-γ, IL-1β, IL-6 and IL-10 levels in the colonic tissue of the animals. *P. peruviana* extract significantly reduced the levels of the pro-inflammatory cytokines TNF-α and IL-1β in the preventive and curative experiments, while the levels of IL-10, IL-6, IL-4 and IFN-γ did not change (Figure 4.7).

4.4.5 RT-PCR

The TNBS-induced colitis produced an increase in the expression of COX-2, iNOS, NLRP3, IL-1β, IL-6, IL-10 and IL-17 genes; while reduction in the MUC2 expression, a protein involved in the epithelial integrity. In the groups treated with *P. peruviana*, a tendency to up-regulate MUC2 and down-regulate COX-2, iNOS, NLRP3, IL-1β, IL-6 and IL-10 expression was observed, without statistical differences (Figure 8). This tendency to the suppression of the genes involved in the inflammatory response can be responsible for the anti-inflammatory activity of the *Physalis peruviana* extract on the TNBS-induced colitis model.

4.4.6 *Physalis peruviana* effect on NO production

The extract of *P. peruviana* did not show toxicity on RAW 264.7 macrophages at concentrations equal or lower than 30 µg/mL (data not shown). This extract significantly inhibited NO release from the macrophages with an IC_{50} of 29.64 µg/mL (95% confidence intervals, 35.27 to 25.11 µg/mL) (Figure 4.9). This reduction of the NO levels in the culture medium of the macrophages treated with *P. peruviana* extract is not related to a NO radical scavenging effect (data not shown).

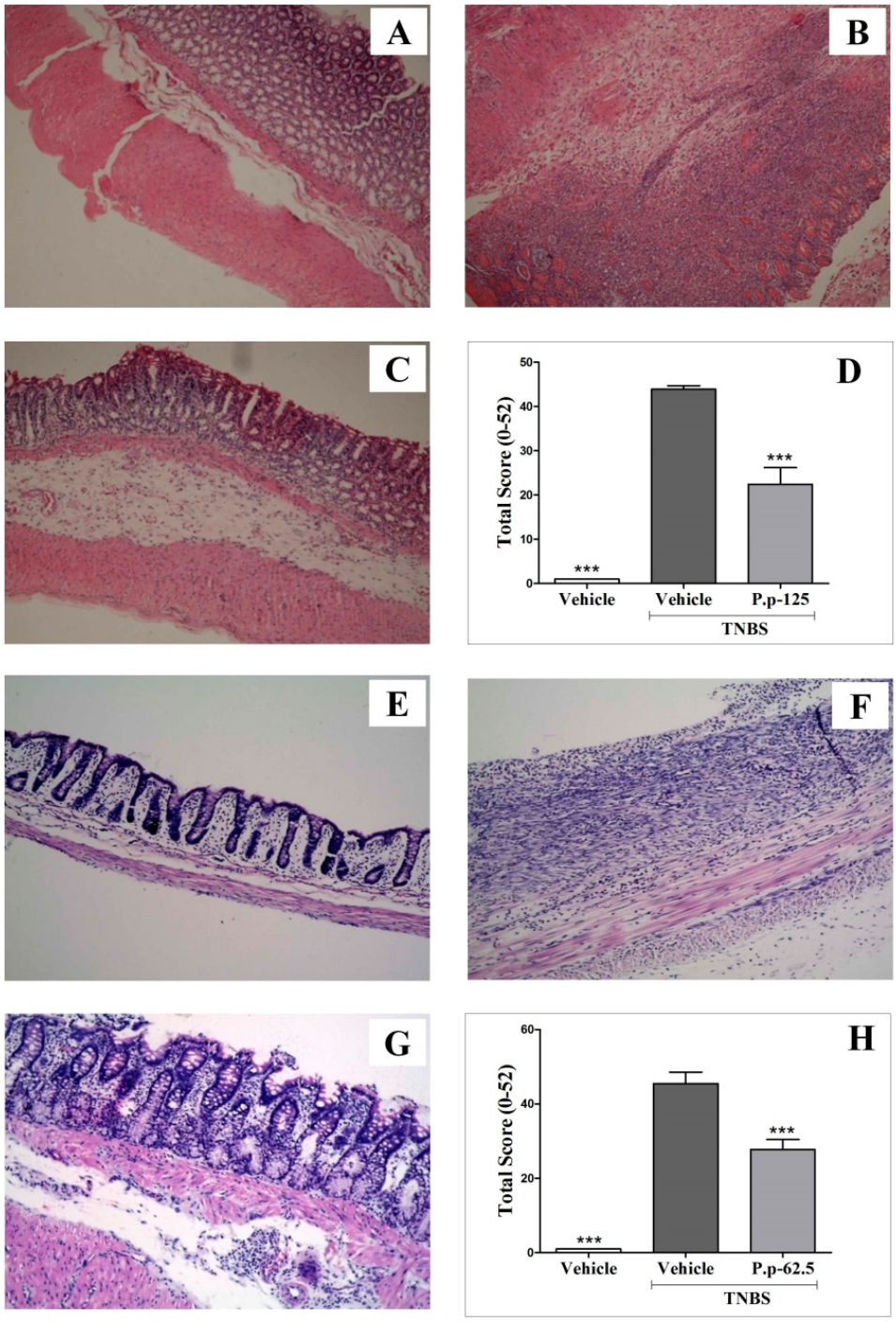


Figure 4.4. Representative histological colonic tissue sections from groups included in the colitis experiment; stained with hematoxylin and eosin. Preventive approach: (A) Sham, (B) TNBS acid and (C) TNBS + *Physalis peruviana* (*P. p*) extract (125 mg/kg/day i.p.). Therapeutic approach: (E) Sham, (F) TNBS acid and (G) TNBS + *Physalis peruviana* (*P. p*) extract (62.5 mg/kg/day i.p.). Pictures were acquired at 10X magnification. The damage score was assigned by a blinded pathologist, using the assessment scale described by Stucchi *et al*. 2000 [32], with some modifications (D and H). The scale presents a score between 0-52, according with the parameters described in Appendix 4. The results represent the mean ± SEM. (n = 10) (***P<0.001 ANOVA, statistically significant against TNBS acid).

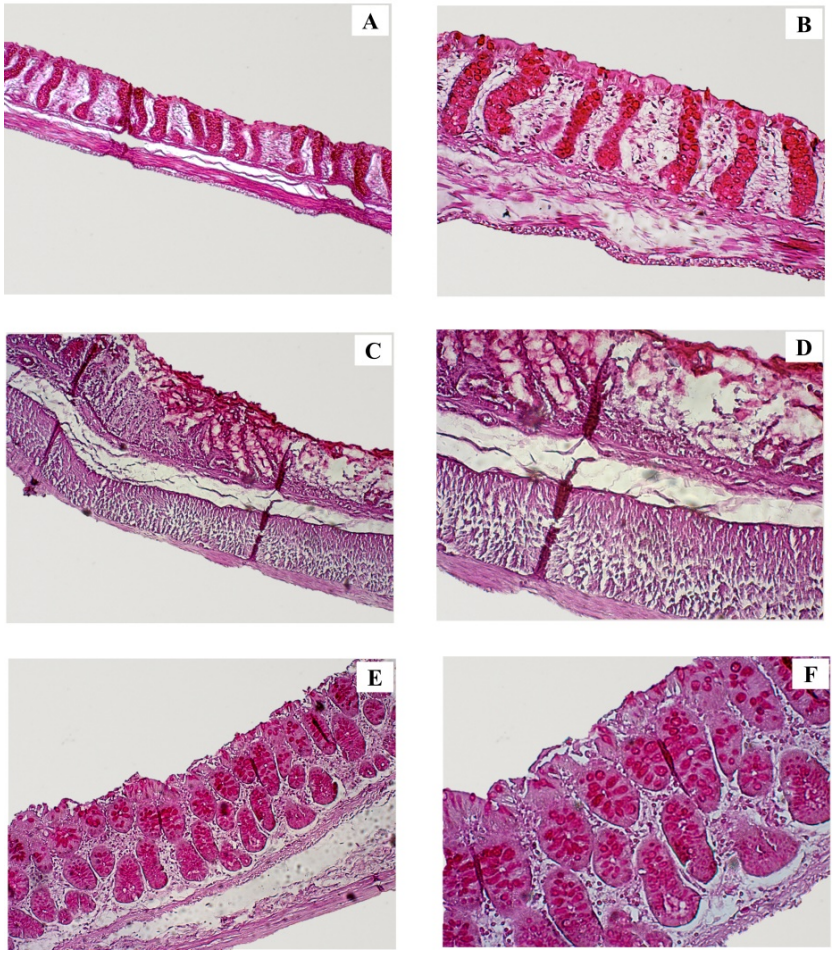


Figure 4.5. Microscopic images of PAS stained colon sections from the groups included in the therapeutic approach. (A and B) Sham, (C and D) TNBS and (E and F) TNBS + *Physalis peruviana* extract (62.5 mg/kg/day i.p.). A blinded pathologist analyzed the sections to determine the number of PAS-positive goblet cells in at least three random fields of 23.54 mm^2. Pictures were acquired at 10X (A, C and E) and 20X (B, D and F) magnifications.

4.4.7 Antioxidant activity

P. peruviana extract did not produce a significant scavenging activity of the DPPH and ABTS radicals with IC_{50} greater than 500 µg/mL in both methods, corresponding to values of 0.76 ± 0.01 and 1.65 ± 0.06 µmol Trolox/g extract, respectively.

4.5 Discussion

The calyx that envelops the fruit of *Physalis peruviana* is formed by sepals or modified leaves that contribute to development and maturation of the fruit, protecting it from insects, birds, diseases and adverse climatic situations.[33, 34] The anti-inflammatory activity showed by the extract of the calyces of *P. peruviana* in the IBD model, might be related with the presence of flavonoids, terpenoids, and glycosides, metabolites found in this organ

which have been reported to affect the production of TNF-α, IL-1β and NO on studies related to IBD.[8] These compounds have also been found in other parts of the plant such as leaves, fruits and roots.[35-38]

The extract obtained from the calyces of *P. peruviana* showed a toxic effect, higher than the observed for leaf extracts in other studies.[38, 39] This elevated toxicity is consequent with the protective function of the plant calyces to safeguard the fruit, and suggests the presence of potent metabolites.[33, 34]

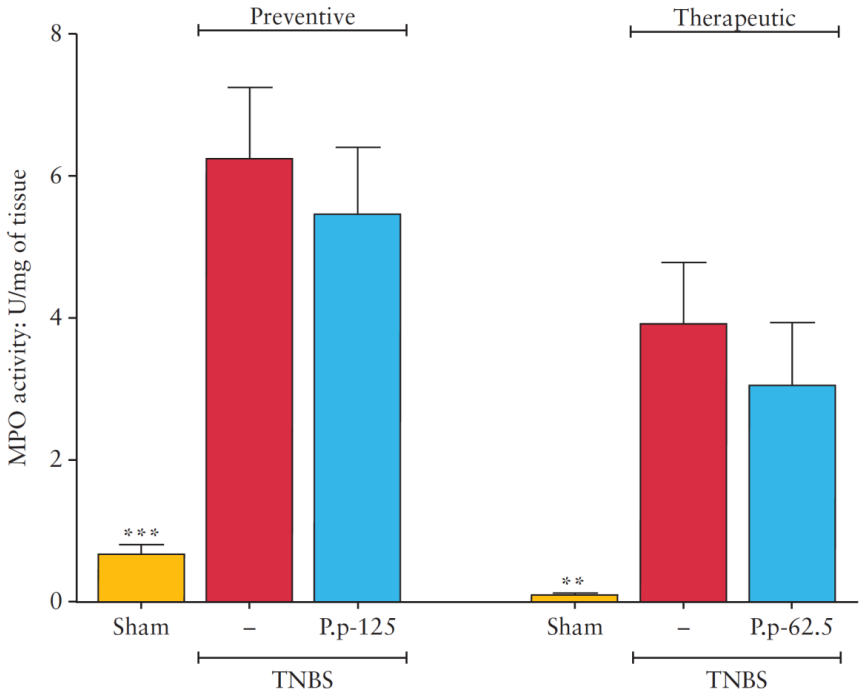


Figure 4.6. Myeloperoxidase (MPO) enzyme activity in colonic segments obtained from the groups included in the TNBS-induced colitis experiment. The results represent the mean ± SEM. (n=10) $^{**}P<0.01$; $^{***}P<0.001$ (ANOVA), statistically significant compared with TNBS acid.

The rectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and ethanol is a chemical model widely employed to produce chronic colonic inflammation with morphologic and histologic alterations that resemble IBD in humans.[40, 41] *P. peruviana* showed a strong anti-inflammatory effect in rats with TNBS-induced colitis, in both the preventive and therapeutic approaches. Macroscopically, the damage score, the extension of damaged areas, and the weight/length ratio were reduced. This last parameter is considered an important and dependable tissue damage index. It shows increases in severe intestinal inflammation, since edema and tissue thickening produce a reduction of the colon length, due to the contraction of the muscular layer of the intestinal wall, as well as the fibrosis presented as a consequence of the constant scarring process in the intestinal mucosa.[42, 43] This beneficial effect was corroborated histologically.

MPO is an enzyme widely distributed in the body. Its principal source is the azurophilic granules of the neutrophils. In IBD, there is an elevated activity of this enzyme, as a result of the recruitment of neutrophils in the intestinal mucosa due to chemokines like CXCL5; therefore, MPO is considered an important biomarker of the infiltration of neutrophils in colonic tissue.[5, 44] The anti-inflammatory activity showed by the *P. peruviana* extract appears to be partially related to its capacity to reduce the infiltration of neutrophils in the colonic tissue, which was also clearly observed in the histological results.

In IBD, macrophages, dendritic cells and T lymphocytes secrete cytokines uncontrollably; which promote the production of reactive oxygen species, NO, prostaglandins, leukotrienes, and others mediators with a consequent loss of the homeostasis that leads to a sustained inflammatory response.[45-47] Similarly, the TNBS administration to the animals produces an increase of TNF-α, IL-1β, IFN-γ, IL-6, IL-10 IL-12, IL-23 and IL-17 levels, while reducing the levels of IL-4 in the colonic tissue.[48-52] *P. peruviana* extract was able to significantly reduce the levels of TNF-α and IL-1β in the colonic tissue, these cytokines share several pro-inflammatory properties that are crucial for the amplification of the response in IBD.**[5]** TNF-α induces the release of pro-inflammatory cytokines as IL-1β. In this sense, it is possible that the reduction of IL-1β levels produced by the *P. peruviana* extract may be related with the inhibition of TNF-α release.[53]

The benefits from the regulation of TNF-α have been proved with the usage of anti-TNF-α drugs, which are effective reducing the inflammation, but these treatments have elevated costs and multiple side effects for the patients.[54] Because of this, the anti-inflammatory activity showed by the *P. peruviana* extract at macroscopic, histologic and biochemical levels, appearing to be closely related to the reduction of the TNF-α production, shows *P. peruviana* as a promising species to obtain new bioactive compounds for the regulation of IBD.

The studies of the expression of genes implicated in the balance of the intestinal immune response have increased the knowledge about the molecular mechanisms associated with the pathogenesis of IBD, and constitute a key strategy to search for new anti-inflammatory agents. In this work, *P. peruviana* showed a tendency to down-regulate the IL-1β expression, suggesting that the extract decreased the levels of this cytokine by suppression of its mRNA levels.

In both IBD and TNBS-induced colitis, there is a decreased production of glycoproteins synthesized by the goblet cells (MUC2).[55] The treatment with *P. peruviana* extract, tends to promote MUC2 expression and the reestablishment the mucus production, compromised by the TNBS rectal instillation, as can be seen in the RT-PCR results and PAS stained colonic tissue.

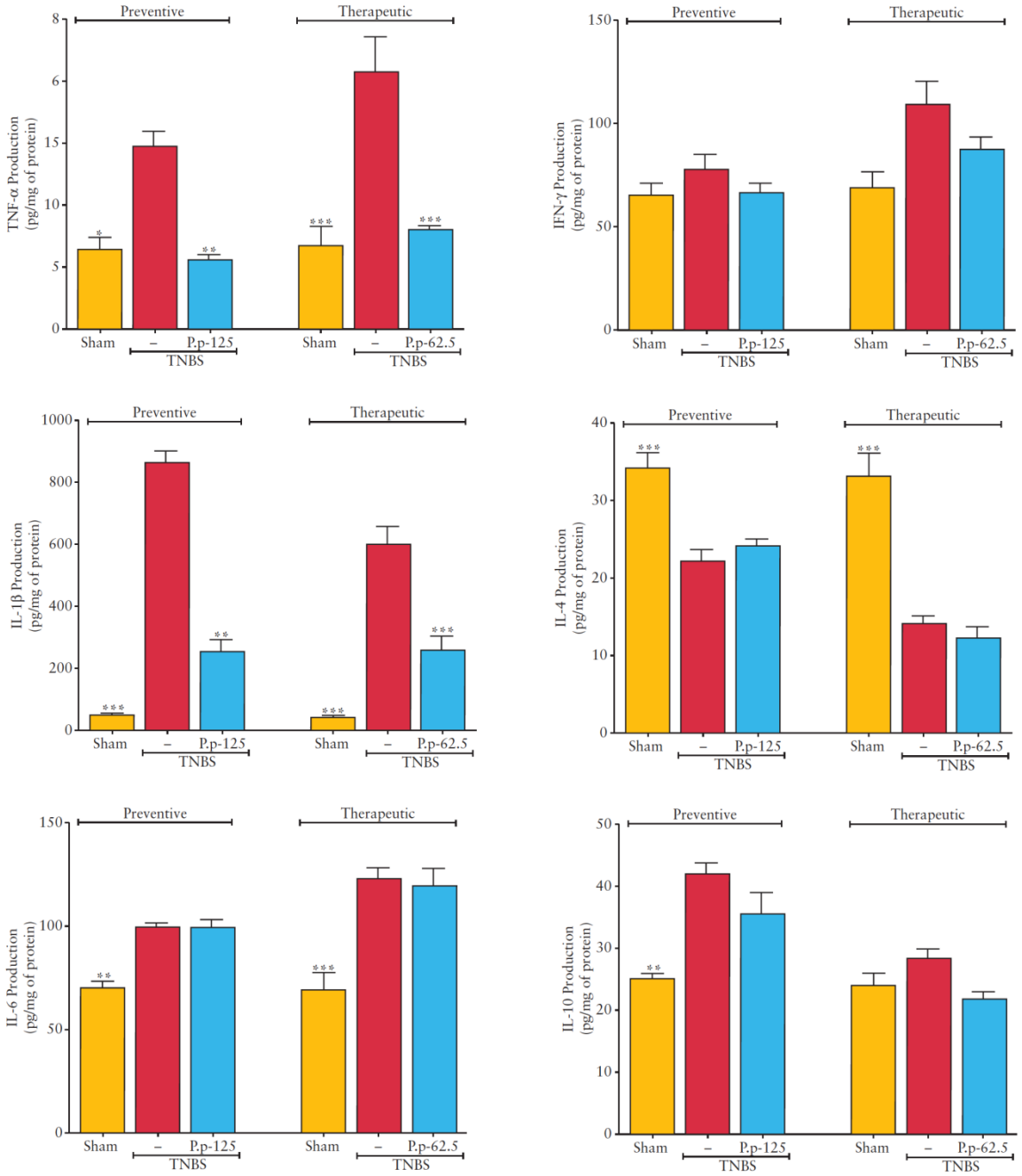


Figure 4.7. *Physalis peruviana* (*P. p*) extract effect on TNF-α, INF-γ, IL-1β, IL-4, IL-6 and IL-10 levels in colonic tissue. Tissue lysates were analyzed using ELISA. The results represent the mean ± SEM. (n=10). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (ANOVA), statistically significant compared with TNBS acid.

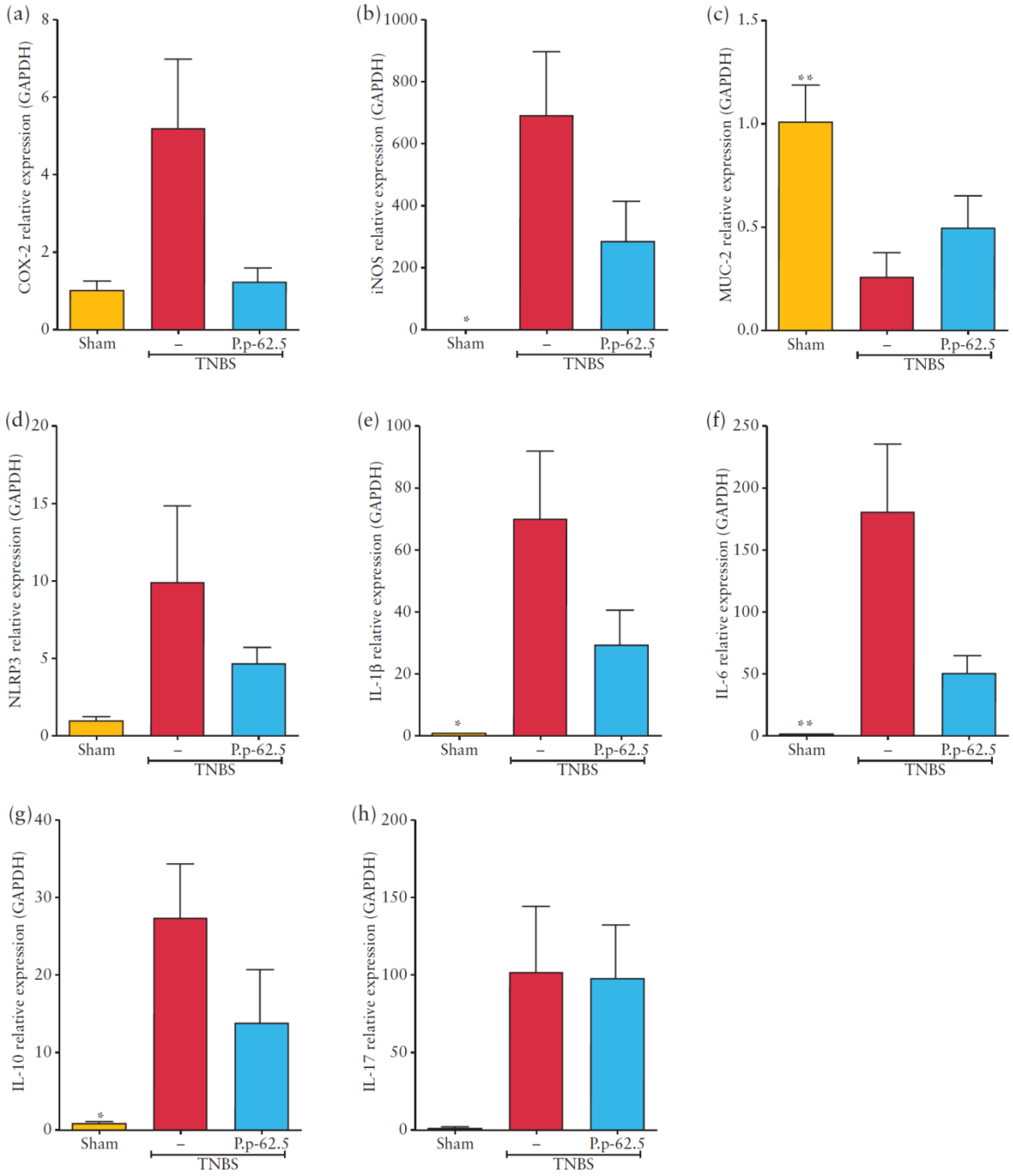


Figure 4.8. Effect of *Physalis peruviana* (*P. p*) extract on the expression of COX-2, iNOS, MUC2, NLRP3, IL-1β, IL-6, IL-10 and IL-17 in colonic tissue from the therapeutic approach experiment. Total mRNA was extracted from the colon and analyzed by RT-PCR. The results represent the mean ± SEM. (n=10) $^{*}P<0.05$; $^{**}P<0.01$ (ANOVA), statistically significant compared with TNBS acid.

The activation of the macrophages RAW 264.7 with LPS triggers characteristic events of acute and chronic inflammatory processes, promoting the synthesis of inflammatory mediators including NO radical.[56] *P. peruviana* extract reduced the NO release in RAW

264.7 macrophages, and showed a trend to down-regulate the iNOS expression in the colonic tissue. Thus, NO seems to be other important target of the anti-inflammatory effect produced by the calyces of this species. The inhibition of the production of this radical has become object of many researches aimed to the development of anti-inflammatory drugs for the treatment of IBD, given the found association between the NO overproduction and the severe damage to the mucosa in this pathology.[57-59]

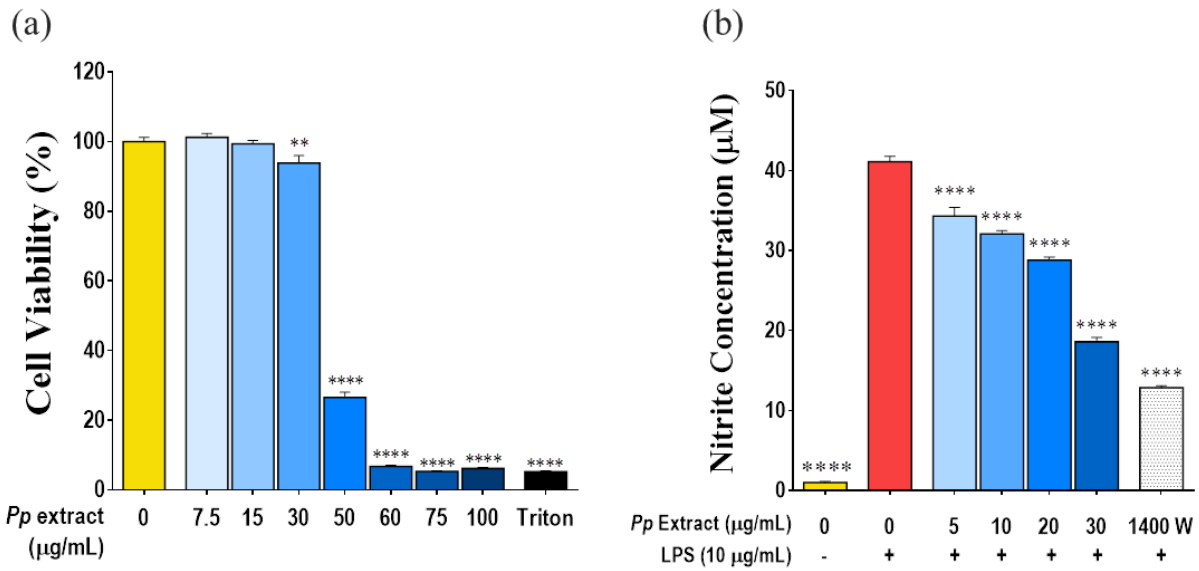


Figure 4.9. Effect of *Physalis peruviana* (*P.p*) extract on the NO release in LPS-stimulated RAW 264.7 macrophages. Cells were cultured with *P.p* extract and activated with LPS. After 24 h, cell viability was evaluated using MTT assay (A) or supernatants collected for nitrite quantification (B). 1400W was employed as positive control. The results represent the mean ± SEM. (n=12). ***P<0.001 (ANOVA), statistically significant compared with LPS (10 µg/mL).

The reactive oxygen species are metabolites associated with the initiation and progression of IBD; control of their synthesis is considered an important target in the search of new therapeutic agents.[60] Gironés-Vilaplana *et al* 2014, reported a hydro-alcoholic extract obtained from the calyces of *P. peruviana* with DPPH and ABTS radical scavenging activity.[61] In our study the total ethereal extract from the calyces of this species did not show a significant scavenging activity on these free radicals, which suggest that the metabolites responsible for this activity are hydrophilic, being more affined to polar phases like the one used by Gironés-Vilaplana. Our results evidence that the strong anti-inflammatory activity displayed by *P. peruviana* extract is not related with free radical scavenging activity.

In conclusion, the total ethereal extract obtained from the calyces of *P. peruviana* significantly reduces the intestinal inflammation induced by TNBS in rats. This activity seems to be related with the reduction of the neutrophils migration into the colonic tissue and restoration of intestinal mucus layer, as well as the decrease of TNF-α, IL-1β and NO

levels, considered crucial targets in IBD. Additional research is needed to deepen in the implicated mechanisms and elucidate the compounds responsible for the anti-inflammatory effect showed by *P. peruviana*.

4.6 References

1. Yamamoto-Furusho, J., et al., *Prevalence and clinical features of indeterminate colitis in Mexico: A 17-year study.* Revista de gastroenterología de México, 2010. **75**(1): p. 30-35.
2. Talley, N.J., et al., *An evidence-based systematic review on medical therapies for inflammatory bowel disease.* The American Journal of Gastroenterology, 2011. **106**: p. S2-S25.
3. Li, M.-C. and S.-H. He, *IL-10 and its related cytokines for treatment of inflammatory bowel disease.* World Journal of Gastroenterology, 2004. **10**(5): p. 620-625.
4. Ko, J. and K. Auyeung, *Inflammatory Bowel Disease: Etiology, Pathogenesis and Current Therapy.* Current pharmaceutical design, 2013.
5. Scaldaferri, F., et al., *Mucosal biomarkers in inflammatory bowel disease: Key pathogenic players or disease predictors?* World journal of gastroenterology: WJG, 2010. **16**(21): p. 2616.
6. Bailón, E., et al., *The intestinal anti-inflammatory effects of the novel agent UR-1505 in the TNBS model of rat colitis are mediated by T-lymphocyte inhibition.* Biochemical pharmacology, 2007. **74**(10): p. 1496-1506.
7. Plevy, S.E. and S.R. Targan, *Future therapeutic approaches for inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1838-46.
8. Hur, S.J., et al., *Review of natural products actions on cytokines in inflammatory bowel disease.* Nutrition Research, 2012. **32**(11): p. 801-816.
9. Rahimi, R., M.R. Shams-Ardekani, and M. Abdollahi, *A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease.* World journal of gastroenterology: WJG, 2010. **16**(36): p. 4504.
10. Wei, J., et al., *Identification of Single-Copy Orthologous Genes between Physalis and Solanum lycopersicum and Analysis of Genetic Diversity in Physalis Using Molecular Markers.* PloS one, 2012. **7**(11): p. e50164.
11. Simbaqueba, J., et al., *Development and characterization of microsatellite markers for the cape gooseberry Physalis peruviana.* PloS one, 2011. **6**(10): p. e26719.
12. Wu, S.-J., et al., *Antioxidant activities of Physalis peruviana.* Biol Pharm Bull, 2005. **28**(6): p. 963-966.
13. Yen, C.Y., et al., *4β-Hydroxywithanolide E from Physalis peruviana (golden berry) inhibits growth of human lung cancer cells through DNA damage, apoptosis and G2/M arrest.* BMC cancer, 2010. **10**(1): p. 46.
14. Martínez, W., et al., *In vitro studies on the relationship between the anti-inflammatory activity of Physalis peruviana extracts and the phagocytic process.* Immunopharmacology and immunotoxicology, 2010. **32**(1): p. 63-73.
15. Franco, L.A., et al., *Actividad antinflamatoria de extractos y fracciones obtenidas de cálices de Physalis peruviana L.* Biomédica, 2007. **27**(1): p. 110-5.
16. Franco, L.A., et al., *Sucrose esters from Physalis peruviana calyces with anti-inflammatory activity.* Planta Med, 2014. **80**(17): p. 1605-1614.
17. Toro, R.M., et al., *Phytochemical analysis, antioxidant and anti-inflammatory activity of calyces from Physalis peruviana.* Nat Prod Commun, 2014. **9**(11): p. 1573-5.
18. Sanabria-Galindo, A., S.I. López, and R. Gualdrón, *Estudio fitoquímico preliminar y letalidad sobre Artemia salina de plantas colombianas.* Rev Col Cienc Quím Farm, 1997. **26**: p. 15-9.

19. Tiwari, P., et al., *Phytochemical screening and extraction: a review.* Internationale Pharmaceutica Sciencia, 2011. **1**(1): p. 98-106.
20. Al-Sultan, S. and Y.A. Hussein, *Acute toxicity of Euphorbia heliscopia in rats.* Pakistan journal of Nutrition, 2006. **5**(2): p. 135-140.
21. Morris, G.P., et al., *Hapten-induced model of chronic inflammation and ulceration in the rat colon.* Gastroenterology, 1989. **96**(3): p. 795-803.
22. Bobin-Dubigeon, C., et al., *Effects of tumour necrosis factor-α synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis.* European journal of pharmacology, 2001. **431**(1): p. 103-110.
23. Castro, J.P., Y.C. Ocampo, and L.A. Franco, *In vivo and in vitro anti-inflammatory activity of Cryptostegia grandiflora Roxb. ex R. Br. leaves.* Biological research, 2014. **47**(1): p. 32.
24. Garrido, G., et al., *In vivo and in vitro anti-inflammatory activity of Mangifera indica L. extract (VIMANG®).* Pharmacological research, 2004. **50**(2): p. 143-149.
25. Whittle, B., et al., *Attenuation of inflammation and cytokine production in rat colitis by a novel selective inhibitor of leukotriene A4 hydrolase.* British journal of pharmacology, 2008. **153**(5): p. 983-991.
26. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Analytical biochemistry, 1976. **72**(1): p. 248-254.
27. Shi, T., et al., *cDNA microarray gene expression profiling of hedgehog signaling pathway inhibition in human colon cancer cells.* PloS one, 2010. **5**(10): p. e13054.
28. Ferrari, M., M.C. Fornasiero, and A.M. Isetta, *MTT colorimetric assay for testing macrophage cytotoxic activity in vitro.* Journal of Immunological Methods, 1990. **131**(2): p. 165-172.
29. Green, L.C., et al., *Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids.* Analytical biochemistry, 1982. **126**(1): p. 131-138.
30. Brand-Williams, W., M. Cuvelier, and C. Berset, *Use of a free radical method to evaluate antioxidant activity.* LWT-Food Science and Technology, 1995. **28**(1): p. 25-30.
31. Re, R., et al., *Antioxidant activity applying an improved ABTS radical cation decolorization assay.* Free radical biology and medicine, 1999. **26**(9): p. 1231-1237.
32. Stucchi, A.F., et al., *NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2000. **279**(6): p. G1298-G1306.
33. Mazorra, M., et al., *Aspectos anatómicos de la formación y crecimiento del fruto de Uchuva Physalis peruviana (Solanaceae).* Acta Biológica Colombiana, 2006. **11**(1): p. 69-81.
34. Puente, L.A., et al., *Physalis peruviana Linnaeus, the multiple properties of a highly functional fruit: A review.* Food Research International, 2011. **44**(7): p. 1733-1740.
35. Mayorga, H., et al., *Glycosidically bound flavor compounds of cape gooseberry (Physalis peruviana L.).* Journal of agricultural and food chemistry, 2001. **49**(4): p. 1904-1908.
36. Mayorga, H., et al., *Hydroxyester disaccharides from fruits of cape gooseberry (Physalis peruviana).* Phytochemistry, 2002. **59**(4): p. 439-445.
37. El-Gengaihi, S.E., et al., *Chemical composition and biological evaluation of Physalis peruviana root as hepato-renal protective agent.* J Diet Suppl, 2013. **10**(1): p. 39-53.
38. Arun, M. and V. Asha, *Preliminary studies on antihepatotoxic effect of Physalis peruviana Linn.(Solanaceae) against carbon tetrachloride induced acute liver injury in rats.* J Ethnopharmacol, 2007. **111**(1): p. 110-114.
39. Kasali, F.M., et al., *Assessment of antidiabetic activity and acute toxicity of leaf extracts from Physalis peruviana L. in guinea-pig.* Asian Pacific journal of tropical Biomedicine, 2013. **3**(11): p. 841-846.

40. te Velde, A.A., M.I. Verstege, and D.W. Hommes, *Critical appraisal of the current practice in murine TNBS- induced colitis.* Inflammatory bowel diseases, 2006. **12**(10): p. 995-999.
41. Brenna, Ø., et al., *Relevance of TNBS-colitis in rats: a methodological study with endoscopic, historical and transcripttomic characterization and correlation to IBD.* PloS one, 2013. **8**(1): p. e54543.
42. Ambrosini, R., et al., *Inflammatory chronic disease of the colon: how to image.* Eur J Radiol, 2007. **61**(3): p. 442-8.
43. Arab, H.H., et al., *Camel's milk ameliorates TNBS-induced colitis in rats via downregulation of inflammatory cytokines and oxidative stress.* Food and Chemical Toxicology, 2014. **69**: p. 294-302.
44. da Silva, M.S., et al., *Anti-inflammatory intestinal activity of Abarema cochliacarpos (Gomes) Barneby & Grimes in TNBS colitis model.* J Ethnopharmacol, 2010. **128**(2): p. 467-475.
45. Sanchez-Muñoz, F., A. Dominguez-Lopez, and J.K. Yamamoto-Furusho, *Role of cytokines in inflammatory bowel disease.* World journal of gastroenterology: WJG, 2008. **14**(27): p. 4280.
46. Algieri, F., et al., *Intestinal anti-inflammatory activity of hydroalcoholic extracts of Phlomis purpurea L. and Phlomis lychnitis L. in the trinitrobenzenesulphonic acid model of rat colitis.* J Ethnopharmacol, 2013. **146**(3): p. 750-9.
47. de Faria, F.M., et al., *Effects of Rhizophora mangle on Experimental Colitis Induced by TNBS in Rats.* Evidence-Based Complementary and Alternative Medicine, 2012. **2012**.
48. Hong, T., et al., *Protective effects of Polygalae root in experimental TNBS-induced colitis in mice.* J Ethnopharmacol, 2002. **79**(3): p. 341-346.
49. Fichtner-Feigl, S., et al., *Cytokines mediating the induction of chronic colitis and colitis-associated fibrosis.* Mucosal immunology, 2008. **1**: p. S24-S27.
50. Randhawa, P.K., et al., *A review on chemical-induced inflammatory bowel disease models in rodents.* The Korean Journal of Physiology & Pharmacology, 2014. **18**(4): p. 279-288.
51. Nascimento Santos Mda, S., et al., *Effect of glucans from Caripia montagnei mushroom on TNBS-induced colitis.* Int J Mol Sci, 2014. **15**(2): p. 2368-85.
52. Arab, H.H., et al., *Telmisartan attenuates colon inflammation, oxidative perturbations and apoptosis in a rat model of experimental inflammatory bowel disease.* PloS one, 2014. **9**(5): p. e97193.
53. Neurath, M.F., *Cytokines in inflammatory bowel disease.* Nat Rev Immunol, 2014. **14**(5): p. 329-42.
54. Pithadia, A.B. and S. Jain, *Treatment of inflammatory bowel disease (IBD).* Pharmacol Rep, 2011. **63**(3): p. 629-642.
55. Kim, Y.S. and S.B. Ho, *Intestinal goblet cells and mucins in health and disease: recent insights and progress.* Current gastroenterology reports, 2010. **12**(5): p. 319-330.
56. Yang, W.S., et al., *Dipterocarpus tuberculatus ethanol extract strongly suppresses in vitro macrophage-mediated inflammatory responses and in vivo acute gastritis.* J Ethnopharmacol, 2013. **146**(3): p. 873-80.
57. Kolios, G., V. Valatas, and S.G. Ward, *Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle.* Immunology, 2004. **113**(4): p. 427-37.
58. Ljung, T., et al., *Rectal nitric oxide as biomarker in the treatment of inflammatory bowel disease: responders versus nonresponders.* World J Gastroenterol, 2006. **12**(21): p. 3386-92.
59. Park, M.Y., et al., *Nitric oxide-induced apoptosis of human dental pulp cells is mediated by the mitochondria-dependent pathway.* Korean J Physiol Pharmacol, 2014. **18**(1): p. 25-32.
60. Debnath, T., H. Kim da, and B.O. Lim, *Natural products as a source of anti-inflammatory agents associated with inflammatory bowel disease.* Molecules, 2013. **18**(6): p. 7253-70.

61. Gironés-Vilaplana, A., et al., *Evaluation of Latin-American fruits rich in phytochemicals with biological effects*. Journal of Functional Foods, 2014. **7**: p. 599-608.

5 CHAPTER FIVE. Protective effect of sucrose esters from Cape gooseberry (*Physalis peruviana* L.) in TNBS-induced colitis

Yanet C. Ocampo[1], Jenny P. Castro[1], Lía Barrios[2], Jesús T. Olivero[1], Virginia Motilva[3], Luis A. Franco[1*]

[1]Biological Evaluation of Promising Substances Group, Faculty of Pharmaceutical Sciences, University of Cartagena, Cartagena, Colombia.
[2]Histopathology Research Group, Faculty of Medicine, University of Cartagena, Cartagena, Colombia.
[3]Department of Pharmacology, Faculty of Pharmacy, University of Seville, Seville, Spain

5.1 Abstract

Phytotherapy constitutes an emerging strategy for Inflammatory Bowel Disease (IBD) treatment that could be especially useful in developing countries. We previously demonstrated the intestinal anti-inflammatory effect of *Physalis peruviana* (Cape gooseberry) calyces in TNBS-induced colitis. This work aimed to investigate the therapeutic potential of peruviose A and B; two sucrose esters which constitute the major metabolites from its calyces. The effect of peruviose A and B mixture on TNBS-induced colitis was studied after 3 (preventive) and 15 days (therapy set-up) of colitis induction in rats. Colonic inflammation was evaluated measuring macroscopic/histologic damage, MPO activity, and biochemical changes. In addition, LPS-stimulated RAW 264.7 macrophages were treated with test compounds. Peruvioses mixture ameliorated TNBS-induced colitis either in acute (preventive) or established (therapeutic) settings. Although, 3-days treatment with compounds did not produced a potent effect, it was sufficient to significantly reduce the extent/severity of tissue damage, and microscopic disturbances. Beneficial effect in the therapy set-up, were significantly higher, and involved the inhibition of pro-inflammatory enzymes (iNOS, COX-2), cytokines (TNF-α, IL-1β, and IL-6), as well as epithelial regeneration with restoration of goblet cells numbers and expression of MUC-2 and TFF-3. Consistently, LPS-induced RAW 264.7 cells produced less NO, PGE2, TNF-α, IL-6, and MCP-1. These effects might be related to inhibition of NF-κB signaling pathway. In conclusion, our results suggest that sucrose esters from *P. peruviana* calyces, non-edible waste from fruit production, might be useful as an alternative herbal treatment for IBD.

Keywords: *Physalis peruviana*, sucrose esters, intestinal inflammation.

5.2 Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, represents a group of chronic, relapsing, and remitting inflammatory conditions of the gastrointestinal tract, that affect millions of people worldwide.[1] Incidence of IBD remains

relatively constant in regions like Northern Europe and North America. Conversely, recent time-trend studies have suggested that IBD is on a rising trend in the areas where incidence was previously low, such as Southern Europe, Asia, and South America.[2]

There is no cure for IBD; however, a variety of treatments are available, including aminosalicylates, corticosteroids, thiopurines, methotrexate, cyclosporine, and biologics such as anti-TNFα therapies.[3] Despite this, current IBD therapies have important shortcomings with regards to safety, efficacy and applicability. Their potentially serious side effects remain a major clinical problem and limit their chronic use. On the other hand, the lack of efficacy is translated into the fact that a large percentage of IBD patients will require intestinal surgery to treat complications at some point following diagnosis.[4] Therefore, novel safe and effective therapeutic strategies are highly desirable.

In this context, the use of herbal medicine to treat IBD is increasing and gaining popularity all around the globe, especially on the account of their low toxicity, biocompatibility, and affordability.[5, 6] This is particularly important in developing countries, where the access of patients to specialized centers and standard therapies is limited.[7] Various natural products have been shown to safely suppress pro-inflammatory pathways and control IBD in both pre-clinical and clinical studies.[8-10] Consequently, the evaluation of extracts obtained from plants traditionally used to treat inflammatory conditions, is an important approach for the development of future therapies for IBD. However, progress in herbal therapeutic research and its clinical application, is limited by the lack of identification of bioactive compounds, insufficient preclinical studies using animal models, and unclear mechanism of action.[5, 11, 12]

Physalis peruviana L. (Solanaceae), commonly known as Cape gooseberry, is native to South American countries including Colombia, Ecuador and Peru.[13] In Colombia, Cape gooseberry is one of the most important exotic fruit crops, representing a source of profit for some regions of the country.[13, 14] With a production of 10.771 tons in 2011, and an export demand of 50%, Colombia is positioned as the largest exporter of this fruit in the world. [15, 16] *P. peruviana* is characterized because its fruit grows enclosed in an inflated calyx, formed by five persistent sepals that develop a 5 cm-size structure that resembles a Chinese lantern. Calyces not only protect the fruit against insects, pathogens or adverse climate; they also play a major role in fruit development; both in size and carbohydrate content.[17] Calyces as well as leaves and small stems are widely employed as traditional herbal remedy medicine for their anti-inflammatory properties.[18].

Despite the folk employment of calyces to treat inflammation, the fruit is still perceived as the only valuable part of the plant, needed for nutritional or medicinal purposes. Indeed, Cape gooseberry is highly appreciated on the international market, particularly in France and Germany, where the fruit is considered a delicacy for its unique flavor, texture and

color.[19] In this sense, calyces from *P. peruviana* are merely employed as decoration for desserts or to increase the shelf life of the fruit after harvesting for exportation to European countries.[16, 20] Otherwise, calyces are removed and discarded, representing the largest portion of the waste generated during Cape gooseberry production.

Recently, we demonstrated that the total ethereal extract from the calyces of *P. peruviana* significantly ameliorates the intestinal inflammation in a murine model of IBD by reducing the production of TNF-α and IL-1β levels.[21] These results established the ability of *P. peruviana* calyces to suppress the altered colon immune response. On the other hand, we found that the major anti-inflammatory metabolites from this ethereal extract were a mixture of sucrose esters, peruvioses A and B.[18, 22] These compounds presented a higher anti-inflammatory activity than their parental extract at lower and well tolerated doses.[18] Thus, in this study, we explored the potential of sucrose esters from *P. peruviana* to modulate the altered inflammatory response in IBD. For this, we employed the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model in rats in two different set-ups: preventive and therapeutic. In addition, we investigated the effect of peruvioses mixture on cytokines production by LPS-activated macrophages, as well as their antioxidant capacity *in vitro*.

5.3 Materials and Methods.

5.3.1 Plant Material

Physalis peruviana L. was collected in La Mesa, Colombia. A voucher specimen (COL-512200) has been deposited at Herbario Nacional Colombiano (Instituto de Ciencias Naturales, Universidad Nacional de Colombia), Bogotá, Colombia.

5.3.2 Sucrose Esters isolation

Isolation of peruviose A and B from *P. peruviana* was carried out as previously described.[18] Briefly, dried calyces (2 Kg) were powdered and extracted with petroleum ether until exhaustion. Total extract (265 g) was partitioned with ether and methanol-water (9:1), and part of the hydroalcoholic fraction (20 g) was further purified by column chromatography on silica gel. The major fraction (8.23 g) was purified by preparative TLC to yield 1100 mg of a mixture of peruviose A and B, occurring in a 6:4 ratio, respectively. Purity of sucrose esters mixture was estimated to be 99.05% by HPLC (LaChrom Elite®, Merck-Hitachi, Darmstadt, Germany) and their structures were confirmed by comparison of their IR and ^{1}H NMR data with those previously reported.[18]

5.3.3 Animals

Six to seven-week-old female Wistar rats (149-195 g) provided by Instituto Nacional de Salud (Bogota, Colombia) were housed in filtered-capped polycarbonate cages containing wood shavings. They were maintained under a 12 h light/darkness cycle in a temperature

(22±3°C) and humidity (65-75%) controlled environment. Animals were allowed to acclimatize for two weeks before use and fed with standard rodent food and water *ad libitum*. Rats were deprived of food 12 h prior to the induction of colitis, but were allowed free access to water throughout. They were randomly assigned to groups of eight to twelve animals in a blinded fashion. Experiments followed a protocol approved by the local Ethics Committee of the University of Cartagena (Minutes of October 14, 2010) and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU).

5.3.4 Sucrose Esters Treatment

Given the low solubility of the mixture of peruviose A and B from *P. peruviana* in water, sucrose esters were co-precipitated with polyvinylpyrrolidone (PVP K-30, USP grade) in a 1:4 (w/w) ratio by solvent evaporation technique. In brief, 6 g of peruvioses mixture were dissolved in 30 mL of dichloromethane and mixed with 24 g of PVP. The resultant solution was dried with a rotatory evaporator with reduced pressure and controlled temperature (40±5ºC). Final solvent removal was done after manual gently scraping of the co-precipitate and drying in oven at 40±5°C overnight. The dried samples were stored in a desiccator at room temperature until use. Sucrose esters were solubilized in phosphate saline buffer (PBS, pH 7.3) and administered intraperitoneally (ip) daily at different doses. The duration and doses of sucrose esters treatment was dependent on the setting of each experiment as detailed below.

5.3.5 Induction of TNBS colitis

Colitis was induced according to the procedure described by Morris *et al.* (1989)[23], with some modifications. Rats were fasted for 12 h and anesthetized with a mixture of 100 mg ketamine (Lab Biosano, Santiago, Chile) and 5 mg of diazepam (Viteco S.A., Bogotá, Colombia) per Kg of body weight. Subsequently, 0.25 mL of TNBS (40 mg/mL; Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol (50 % v/v) was instilled rectally using a cannula (external diameter 2 mm) introduced to an 8 cm depth. A healthy control group was included which received saline in a comparable volume. Following the instillation of the hapten, the animals were maintained in a head-down position until they recovered from anesthesia to prevent leakage of the intracolonic instillate.

Two different protocols were designed to study the anti-inflammatory effect of peruviose A and B mixture. In the first approach rats were preventively treated with sucrose esters (20, 10, and 5 mg/kg/day, ip) 48, 24 and 2 h before colitis induction as well as 24 h thereafter, and were sacrificed 48 h after colitis induction. To evaluate the therapeutic effect sucrose esters (10 and 5 mg/kg/day, ip) were administered after TNBS instillation and daily for two weeks before animal sacrifice. Control and TNBS groups were also treated with vehicle (PVP K-30 dissolved in PBS) by ip route. In both outlines, animal body weight, occurrence of diarrhea, and food intake were monitored daily. Once the animals were sacrifice by

cervical dislocation, the colon was removed, cleaned, longitudinally opened, and its weight/length ratio was recorded. Each colon was scored for macroscopically visible damage by measuring the extent (cm^2) and severity of the lesions (score 0-10) in the distal colon as well as the presence of adhesions (score 0–2), according to the criteria of Bobin-Dubigeon *et al*, 2001.[24] Representative colon, liver and kidney samples were collected and fixed in buffered formalin for the histological analysis. Pieces of the colon were collected, and either placed in RNA Later® (Qiagen, Valencia, CA, USA) or frozen in liquid N_2, immediately on collection and stored at -80°C until the measurement of biochemical parameters or RNA extraction was performed.

5.3.6 Histology Analysis

Paraffin-embedded colon samples were sliced at 5 μm, and stained with hematoxylin and eosin (H&E), or periodic acid Schiff (PAS), according to standard protocols, for histological evaluation of colonic damage and mucus content. Samples were blindly analyzed by an experienced pathologist employing light microscopy (Olympus BX41, Tokyo, Japan). The histological damage was evaluated employing the scoring system described by Arribas *et al*, 2001 (Appendix 4).[25] The extent of mucus production was estimated by determining the number of PAS-positive goblet cells in at least three fields of 23.54 mm^2, randomly selected from each slide at 20X magnification. Liver and kidney samples were stained with H&E and evaluated by pathologist (Appendix 4). Images were captured at 10X and 40X magnifications on a Zeiss Axio Lab.A1 microscope (Carl Zeiss, Oberkochen, Germany), coupled to a computer driven Zeiss AxioCAM digital camera (ICc5).

5.3.7 MPO Activity Assay

The enzyme activity was measured according to the technique described by Castro *et al.* 2014,[26] and the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 mmol hydrogen peroxide/min at 25 °C.[27]

5.3.8 Measurement of cytokine levels

Colon biopsies were homogenized in Greenburger buffer pH 7.4, containing protease inhibitors (cOmplete Mini EDTA free; Roche, Basel, Switzerland), sonicated for 10 sec, and centrifuged at 10.000 rpm and 4ºC for 10 minutes. Homogenates were stored at -20°C until use. The levels of INF-γ, IL-1β, IL-4, IL-6, IL-10, and TNF-α, were quantified by ELISA (Invitrogen, Carlsbad, CA, USA or R&D Systems, Minneapolis, MN, USA), according to the manufacturers protocol. Cytokine levels were normalized using the total quantity of proteins as determined by Bradford method (Bio-Rad, Hercules, CA, USA).

5.3.9 Quantitative Real-time PCR (RT-PCR)

Total mRNA was isolated from colon tissue using the RNeasy® kit (Qiagen, Valencia, CA, USA) as described by the manufacturer. RNA was quantified and purity assessed by

determining the 260/280 nm absorbance ratio using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). For each sample, 2.0 µg of RNA were employed as template for cDNA synthesis using the Quantitect reverse transcription kit (Qiagen, Valencia, CA, USA). Real-time PCR analysis was performed using the LightCycler® 96 System (Roche, Mannheim, Germany) with Power SYBR® Green PCR master mix (Applied Biosystems, Forsters, CA, USA) and specific primers (Eurofins Genomics, Huntsville, AL, USA), according to the manufacturer's instructions. Primer sequences were obtained from literature and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Appendix 5). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous housekeeping gene. Duplicate cycle threshold (CT) values were analyzed by the comparative CT (ΔΔCT) method.

5.3.10 Western Blot

Colon tissue was homogenized and cytoplasmic/nuclear proteins were isolated with the ReadyPrep protein extraction kit (Biorad, Hercules, CA, USA) according to the manufacturer´s instructions. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Aliquots of 30 µg proteins were fractionated by SDS-PAGE (7.5% tris-glycine-polyacrylamide gels) and transferred to Immobilon®-P membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% milk in 0.1% Tween-20-PBS for 1 h and incubated overnight with primary antibodies. Antibodies for β-actin (sc-47778), iNOS (sc-7271) and NF-κB p50 (sc-166588), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and used at a dilution of 1:200 to 1:500. The membranes were washed with 0.1% Tween 20-PBS and then incubated for 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed again and incubated with chemiluminescent substrate (Clarity^{TM} Western ECL Substrate; Bio-Rad, CA, USA) for 5 min. The membranes were captured using the G:BOX imager (Syngene, Cambridge, UK), and proteins bands quantified with the GeneTools analysis software (Syngene, Cambridge, UK).

5.3.11 Cell culture

RAW 264.7 macrophages were obtained from the American Type Culture Collection (TIB-71; Rockville, MD, USA) and routinely cultured in Dulbecco's Modified Eagle Medium-high glucose (DMEM) supplemented with 2 mM L-glutamine, antibiotics (100 UI/mL of penicillin-100 µg/mL streptomycin) and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

5.3.12 MTT reduction assay

Cytotoxic effect of test compounds was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT assay).[28] RAW 264.7 cells were cultured

into 96-well plates (2 x10^5 cells/mL) and allowed to grow to confluence. Subsequently, the culture medium was discarded and replaced with sucrose esters (0-100 µg/mL) or ethanol vehicle for 30 minutes, followed by stimulation with 10 µg/mL lipopolysaccharide (LPS). After 24 h, supernatant was replaced by fresh medium containing MTT (0.2 mg/mL). Four hours later, the medium was carefully aspirated and formazan crystals were dissolved in DMSO (100 µL), and the OD_{550} was measured using a microplate reader. The viabilities of treated cells were expressed as the percentage of control cells, which was assumed to be 100%.

5.3.13 Macrophages stimulation assay.

RAW 264.7 macrophages were treated in a manner similar to that described for cell viability. In brief, cells were seeded in 24-well plates (2x10^5 cells/mL) and treated for 30 min with various concentrations of sucrose esters (0-20 µg/mL), N-(3-(aminomethyl) benzyl)acetamidine (1400W, 2.50 µg/mL), rofecoxib (3.14 µg/mL) or dexamethasone (3.92 µg/mL), and stimulated with LPS (10 µg/mL). Control cells were cultured under the same conditions but were not exposed to the effect of LPS. Twenty-four hours later, culture supernatants were collected and stored at -20°C until use.

NO production was estimated from the accumulation of NO_2^- in the medium using the Griess reagent, as described previously.[29] Briefly, equal volumes of supernatants and Griess reagent (100 µL) were mixed and incubated at room temperature for 5 minutes. The OD_{550} of the samples was measured using a microplate reader. The amount of nitrite in the samples was calculated from a standard curve (0-200 uM) of sodium nitrite ($NaNO_2$). Levels of IL-1β, IL-6, MCP-1, PGE2, and TNF-α, in culture supernatants were determined using ELISA (R&D Systems, Minneapolis, MN, USA or eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Final results were expressed as pg of mediator/mL of supernatant.

5.3.14 Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM) for each group. One-way analysis of variance (ANOVA), followed by Tukey *post hoc* test, was used to determine differences between treatment groups. Kaplan–Meier analysis with log-rank statistics was performed in survival curve during TNBS-induced colitis. Values of $P<0.05$ were considered significant.

5.4 Results

5.4.1 Intestinal anti-inflammatory effect of peruvioses in TNBS-induced colitis.

In the preventive approach, Wistar rats were treated with the mixture of peruviose A and B from *Physalis peruviana* two days before rectal instillation of TNBS. Three days after colitis induction, the severity of intestinal inflammation was assessed by macroscopic,

histological, and biochemical parameters (Figure 5.1). Rats treated with TNBS showed hypoactivity, piloerection, and developed diarrhea associated with reduction in food intake (data not shown). Consequently, animals were severely anorexic with a substantial decrease in body weight in comparison to control group (P<0.0001) (Figure 5.1B). Correspondingly, macroscopic inspection of the colon showed evidence of severe inflammation characterized by necrosis of the mucosa, extending 5-10 cm^2 along the tissue, edema, hyperemia, deep ulcerations and focal adhesion to adjacent organs. Although the pretreatment with sucrose esters did not modify food intake or body weight loss, compounds produced a significant anti-inflammatory effect when administrated at doses of 10 and 20 mg/Kg/day, ip. As shown in Figure 5.1C, sucrose esters treated rats presented less severe and extended inflammation of the colon showing a reduction of macroscopic damaged area of at least 28.50% (P<0.05 vs. TNBS group). Conversely, the weight/length ratio of the rat colon was not significantly reduced at these doses.

Microscopically, colon tissue from TNBS group was characterized by extensive ulceration and necrosis of mucosa, typically affecting over 75% of the surface, accompanied by massive depletion of goblet cells, transmural inflammation with edema and prominent infiltration of inflammatory cells at all the intestinal layers. In this group, the grade of lesion was considered severe, with a score of 39.73±3.09 (P<0.0001 vs. control group, Figure 5.1D). Consistently, colonic injury induced by TNBS was characterized by an enhancement in myeloperoxidase (MPO) activity compared to control group (P<0.0001, Figure 5.1E), suggesting large neutrophil infiltration. Treatment with peruvioses mixture (10 and 20 mg/Kg) diminished significantly the histological score by over 35% (P<0.05 vs. TNBS group, Figure 5.1D) with dose-dependent reduction of mucosal ulceration, edema, number of infiltrating cells, and restoration of the colonic architecture as well as slight improvement of goblet cells numbers. Despite these observations, we found that MPO activity was not diminished significantly (Figure 5.1E).

To further characterize the beneficial effect of sucrose esters in TNBS-induced colitis, rats were treated with peruvioses A and B (5 and 10 mg/Kg/day) for 2 weeks after TNBS instillation, in a therapeutic set up of established colitis (Figure 5.2A).[30] TNBS-induced colitis produced signs of abdominal pain, hypoactivity, piloerection and a mortality rate exceeding 37% (P<0.05 vs. control group), whereas animals that received higher dose of sucrose esters (10 mg/Kg) showed a slight improvement in mortality rates (20%, Figure 5.2B).

Immediately after TNBS instillation, rats developed diarrhea and reduction of food intake that was accompanied by a striking loss of body weight. As expected, the body weight of the rats with colitis remained significantly lower than that of the control animals for the duration of the study (P<0.05, Figure 5.2C). On the other hand, five to six days after TNBS instillation, sucrose esters treated animals started to recover from the weight loss, returning

to baseline body weight by day eight and increasing it significantly by the end of the experiment ($P<0.05$, vs. TNBS group). We also monitored the effect of sucrose esters in food intake and found a trend of recovery consistent with changes in body weight (data not shown). Thus, treatment with peruviose A and B mixture resulted in improved survival rate, in addition to reducing the manifestations of TNBS-induced colitis.

Macroscopic damage induced by TNBS was characterized by adhesion to adjacent organs, and mild ulceration, with a damaged area extending 7-24 cm^2 along the tissue. Furthermore, we found severe bowel wall thickening, with tissue having a hard and rigid texture suggesting fibrosis, as well as visible strictures, occasionally associated with obstruction (Figure 5.2D). The treatment with sucrose esters from *P. peruviana*, reduced the extension and severity of macroscopic damage induced by TNBS ($P<0.01$), producing a recovery of tissue elasticity, texture and thickness, as recorded by blinded observers. Consistently, the weight/length ratio of rat colon was significantly diminished at both doses (Figure 5.2E, $P<0.05$ vs. TNBS group).

At the histological level, colon tissue from TNBS group presented epithelial ulceration, affecting in average more than 50% of the surface, and complete necrosis of mucosa in 40% of analyzed slides, as well as transmural inflammation with profuse infiltration of inflammatory cells, and hyperplasia of all the intestinal layers. The inflammatory process was associated with crypt hyperplasia and substantial goblet cell depletion. In this group, the grade of lesion was considered severe, with a score of 44.00±2.98 ($P<0.0001$ vs. control group). As expected, MPO activity was significantly increased in comparison to control animals ($P<0.001$).

Treatment with the mixture of peruviose A and B (5 and 10 mg/Kg) produced a significant recovery of the damage induced by TNBS, giving a total score of 15.43±3.93 and 16.67±9.31 (Figure 5.2G-1, $P<0.01$), respectively, graded as mild by the pathologist. Most of the samples showed a remarkable restoration of the epithelial cell layer and crypt architecture, with less than 16% of analyzed tissue showing complete necrosis of mucosa. The transmural involvement, inflammation, edema, hyperplasia, as well as goblet cell depletion was significantly less severe, in contrast to animals of TNBS group. Additionally, the histological improvement was accompanied by a significant reduction of infiltration of inflammatory cells. MPO activity was reduced by over 75% (Figure 5.2F, $P<0.05$).

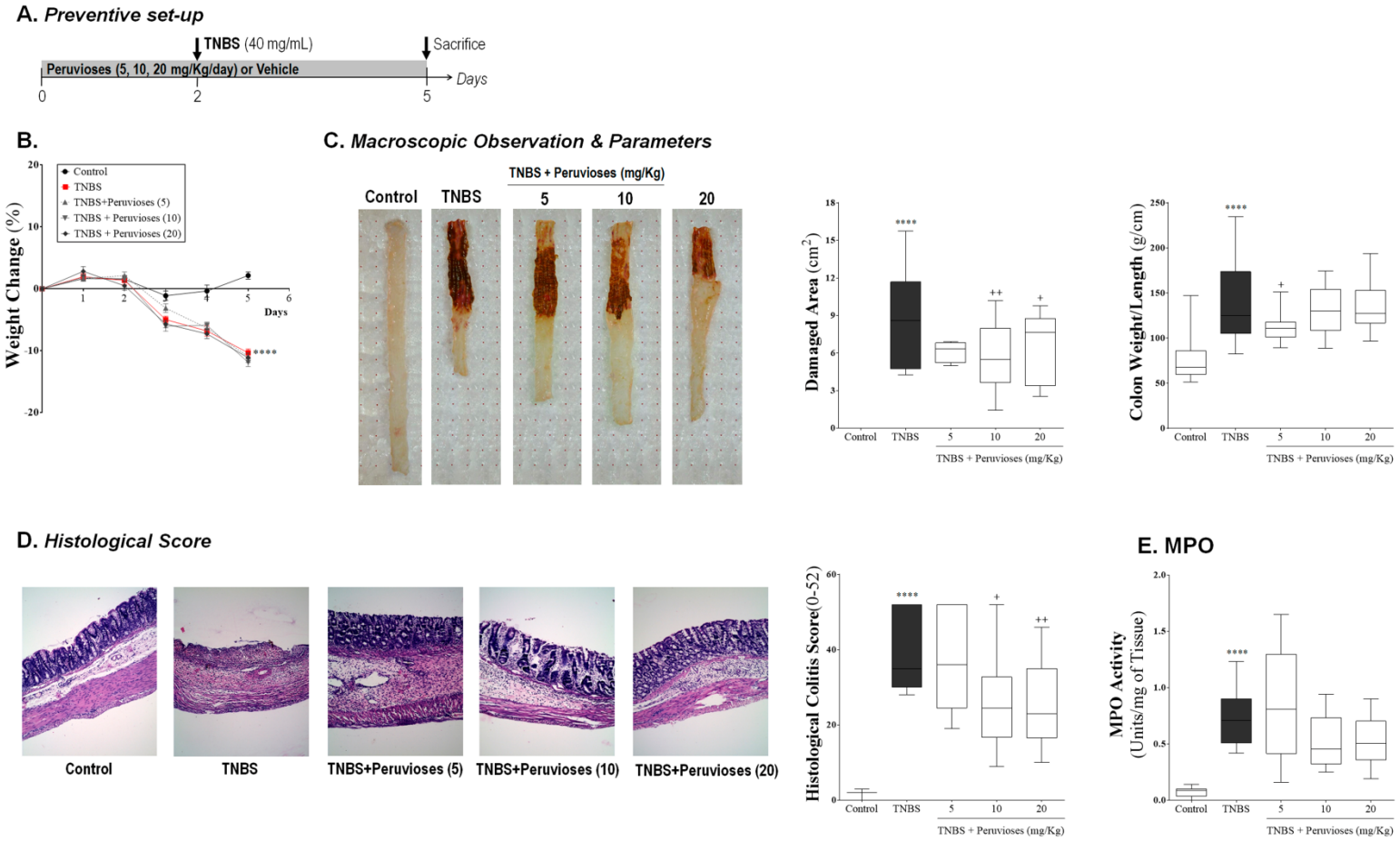


Figure 5.1. Pre-treatment with peruvioses A and B from *Physalis peruviana* ameliorates acute TNBS-colitis. (A) Wistar rats were treated for 2 days with test compounds (5, 10, and 20 mg/Kg/day, ip) or vehicle. Colitis was induced by instillation of TNBS. Three days thereafter, animals were sacrificed and colitis severity was assessed. **(B)** Animals weight change. **(C)** Appearance, damaged area (cm^2), and colon weight/length. Representative pictures of rat colon are shown. **(D)** Histologic changes were examined by H&E staining and scored by a blinded pathologist, according to the parameters described in Appendix 4. Representative pictures are shown (10X). **(E)** MPO activity was measured in colon biopsies. Results are representative of at least two independent experiments, and are expressed as the mean ± SEM. (n=11-20 per group). (****) P<0.0001 vs. control; (+) P<0.05 and (++) P<0.01 vs. TNBS group.

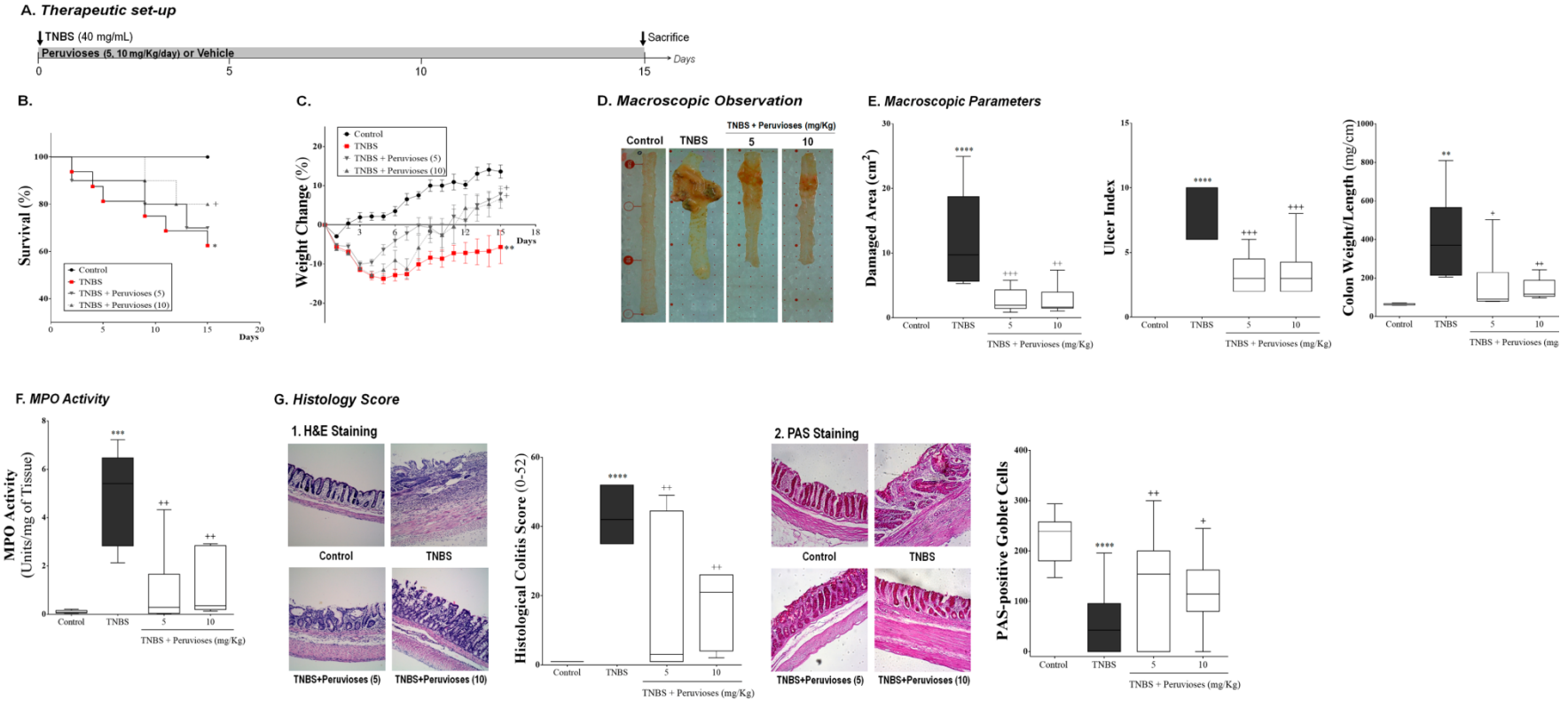


Figure 5.2. Peruvioses A and B from *Physalis peruviana* diminished the inflammation induced by TNBS. (A) Colitis was induced in Wistar rats by instillation of TNBS. Animals were treated with peruvioses mixture (5 and 10 mg/Kg/day, ip) or vehicle for 15 days. Afterwards, rats were sacrificed and colitis severity was assessed. **(B)** Survival and **(C)** body weight changes were monitored on daily basis. **(D)** Appearance of colon tissue was evaluated and representative pictures are shown. **(E)** Macroscopic damage (damaged area (cm^2), ulcer index, and colon weight/length) were scored as detailed in Materials and Methods. **(F)** Myeloperoxidase (MPO) activity was measured in colon biopsies. **(F)** Histological changes were examined after (1) hematoxylin and eosin (H&E) and (2) Periodic Acid Schiff (PAS) staining. Slides were examined by a blinded pathologist, according to the parameters described in Appendix 4. Representative pictures are shown, magnification 10X (H&E) and 20X (PAS), respectively. Results are representative of at least two independent experiments, and are expressed as the mean ± SEM (n=10-24 per group). (*) P<0.05, (**) P<0.01, (***) P<0.001, and (****) P<0.0001 vs. control; (+) P<0.05, (++) P<0.01, and (+++) P<0.001 vs. TNBS group.

To confirm the beneficial effect of sucrose esters in goblet cells recovery and their mucin production, the PAS staining technique was employed. In TNBS group, the proportion of goblet cells containing neutral mucins (PAS-positive cells) was lower than in control animals (P<0.0001). We observed that PAS-positive cells were larger, nearly absent at the surface epithelium and strongly reduced in areas were crypts abnormalities were more severe (Figure 5.2G-2). In contrast, rats treated with sucrose esters mixture from *P. peruviana* (5 and 10 mg/Kg), showed a remarkable increase of PAS-positive cells by over 2 fold (P<0.05 vs. TNBS group) (Figure 5.2G-2). The increased number of PAS-positive cells suggests that peruvioses A and B are promoting tissue repair mechanisms.

5.4.2 Peruviose A and B mixture showed a safe toxicological profile.

A follow up of the animals treated with sucrose esters from *P. peruviana* calyces, in both preventive and therapeutic experiments, allowed us to evaluate the safety of tested compounds. Within ∼1–2 min after administration of the mixture of peruvioses A and B (20 mg/Kg/day; ip), animals assumed a recumbent posture and exhibited piloerection and occasional abdominal writhes, that peaked 2-5 minutes after injection and declined abruptly thereafter. Besides these adverse effects of pain/distress, no mortality or signs of toxicity were observed when compounds were administered at this dose for 5 days. On the other hand, this reaction to test compounds was not observed with lower doses of sucrose esters (10 and 5 mg/Kg/day; ip), when administered consecutively either for 5 or 15 days.

During necropsy, a macroscopic examination did not show detectable changes in the morphology of liver and kidneys due to the administration of peruvioses A and B (20, 10 or 5 mg/Kg) in both experiments. In addition, the histopathology evaluation did not reveal significant changes in tissue architecture of these organs in comparison to the control group (data not shown).

5.4.3 Peruviose A and B reduce inflammatory gene and protein expression induced by TNBS instillation.

In the preventive study, TNBS administration promoted a significant increase of iNOS, COX-2, IL-1β, IL-6, and IL-10 expression (P<0.05 vs. control group), as well as a major reduction in MUC-2 (P<0.05 vs. control group). The administration of sucrose esters mixture from *P. peruviana* inhibited dose-dependently the expression of iNOS and COX-2 (P<0.05), while failing to modulate changes in the rest of evaluated genes (Figure 3A). On the other hand, ELISA analysis confirmed that TNBS boosted the production of IL-1β, IL-6, IL-10 and TNF-α, and reduced IL-4 levels (Figure 5.3B, P<0.001 vs. control group). Additionally, it demonstrated that animals treated with sucrose esters from *P. peruviana* produced a significant reduction of TNF-α and IL-10 (Figure 5.3B; P<0.05 vs. TNBS group), whereas the overproduction of other cytokines was not modified.

As can be seen in Figure 5.4A and 5.4B, the colonic inflammation induced by TNBS in the therapeutic setup was characterized by a prominent up-regulation of mRNA expression of iNOS, COX-2, IL-1β, IL-6, IL-10, and IL-17A (P<0.05 vs. control group). As expected, treatment with peruvioses A and B mixture (5 and 10 mg/Kg/day, ip) inhibited strongly the expression of iNOS and COX-2 compared to TNBS rats (P<0.05). When the production of NO in colon tissue homogenates was evaluated (Figure 5.5B), we found that the treatment with sucrose esters resulted in a marked decrease of the levels of this mediator, in comparison with TNBS group (60% inhibition, P<0.001). Moreover, western blot assays confirmed the reduction of iNOS expression at the protein level (Figure 5.5C) in rats treated with TNBS and the mixture of peruvioses.

On the other hand, the transcriptional levels of all the evaluated cytokines (IL-1β, IL-6, IL-10, and IL-17) were also significantly down-regulated in sucrose esters treated animals (P<0.05 vs. TNBS group). Furthermore, ELISA analysis (Figure 5.5A) confirmed that compounds were able to reduce the production of TNF-α, IL-1β, and IL-10 at the protein level (P<0.05 vs. TNBS group), whereas the levels of IL-6, IFN-γ, and IL-4 were not modified. Accordingly, the RT-PCR analysis (Figure 5.4D) showed a significant reduction in the expression of the nuclear transcription factor kappa B (NF-κB), in TNBS rats treated with the mixture of peruvioses (P<0.05 vs. TNBS group). The blockage of NF-κB activation and nuclear translocation was verified with western blot (Figure 5.5C).

TNBS prompted an important reduction in MUC-2 expression compared to control animals (P<0.05). Treatment with sucrose esters (5 and 10 mg/Kg) increased the expression of MUC-2 back to the levels of control animals. Since, the building block of intestinal mucus is MUC-2, a gel forming mucin secreted by goblet cells, and taking into account our histological findings, we hypothesized that sucrose esters from *P. peruviana* might be stimulating the recovery of the colonic epithelium, thus enhancing goblet cells production of mucins. To test this, we further evaluated the expression of genes involved in mucus/mucosa integrity. Peruvioses A and B demonstrated a beneficial impact in restoring the expression of TFF-3 (P<0.05) (Figure 5.4C). In the case for MUC-3, a tendency for higher expression in sucrose esters treated animals was also observed.

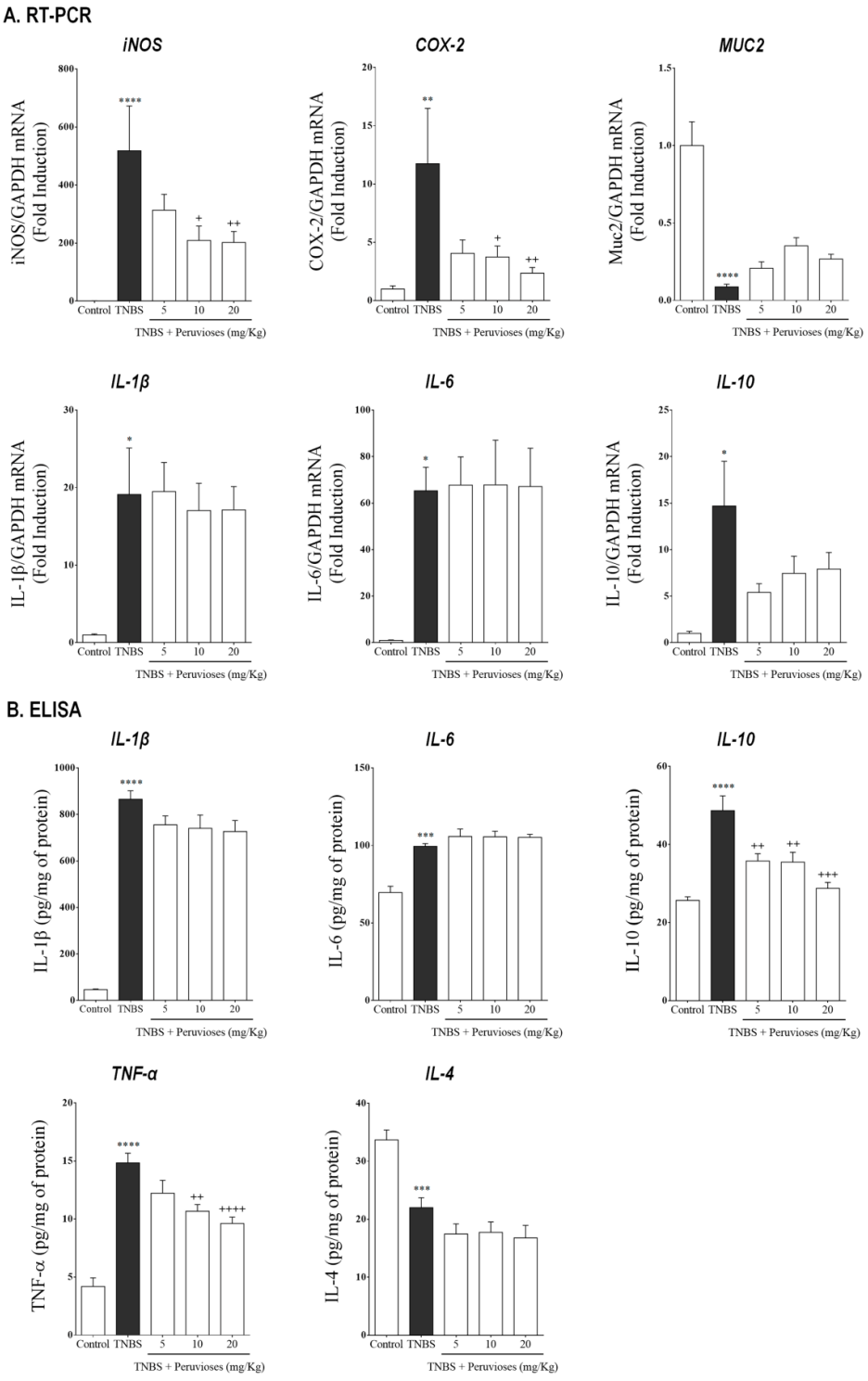


Figure 5.3. Peruvioses A and B pre-treatment reduce the expression of pro-inflammatory enzymes (iNOS, COX-2) and cytokines (TNF-α, IL-10) in acute TNBS-colitis. (A) iNOS, COX-2, MUC-2, IL-1β, IL-6, and IL-10 mRNA expression was quantified by RT-PCR. **(B)** IL-1β, IL-6, IL-10, TNF-α, and IL-4 protein levels were measured by ELISA. Results are representative of at least two independent experiments, and are expressed as the mean ± SEM (n=9-15 per group). (*) P<0.05, (**) P<0.01, (***) P<0.001, and (****) P<0.0001 vs. control; (+) P<0.05, (++) P<0.01, (+++) P<0.001, and (++++) P<0.0001 vs. TNBS group.

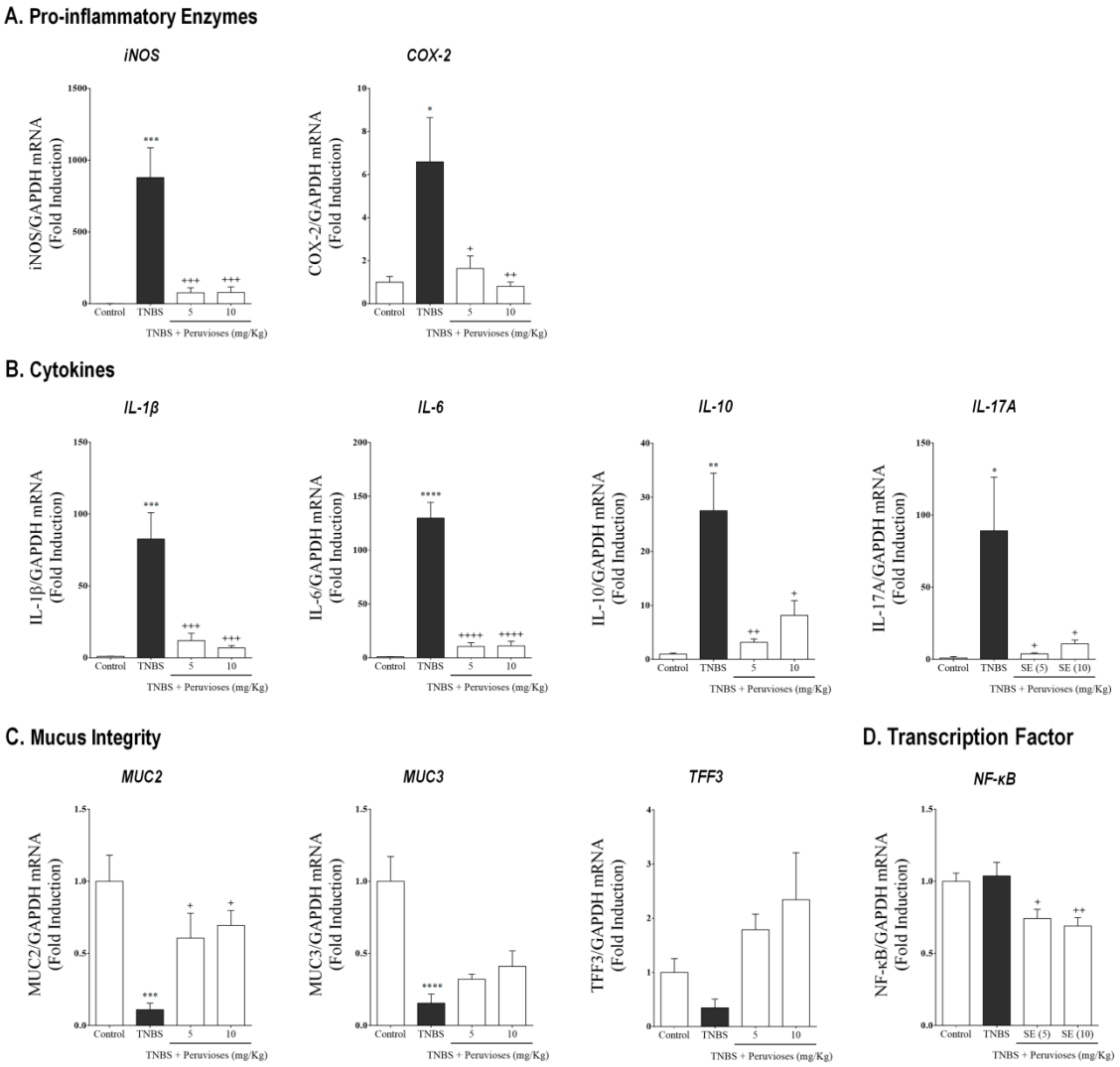


Figure 5.4. Treatment with peruvioses A and B modulates the expression of pro-inflammatory enzymes, cytokines, and NF-κB expression and restores markers of mucus integrity hampered in rats with established TNBS-induced colitis. (A) Pro-inflammatory enzymes (iNOS, COX-2), **(B)** Cytokines (IL-1β, IL-6, IL-10, and IL-17B), **(C)** Mucus integrity markers (MUC-2, MUC-3, and TFF-3), and the transcription factor NF-κB mRNA expression was quantified by RT-PCR. Results are representative of at least two independent experiments, and are expressed as the mean ± SEM (*n*=7-10 per group). (*) *P*<0.05, (**) *P*<0.01, (***) *P*<0.001, and (****) *P*<0.0001 vs. control; (+) *P*<0.05, (++) *P*<0.01, (+++) *P*<0.001, and (++++) *P*<0.0001 vs. TNBS group.

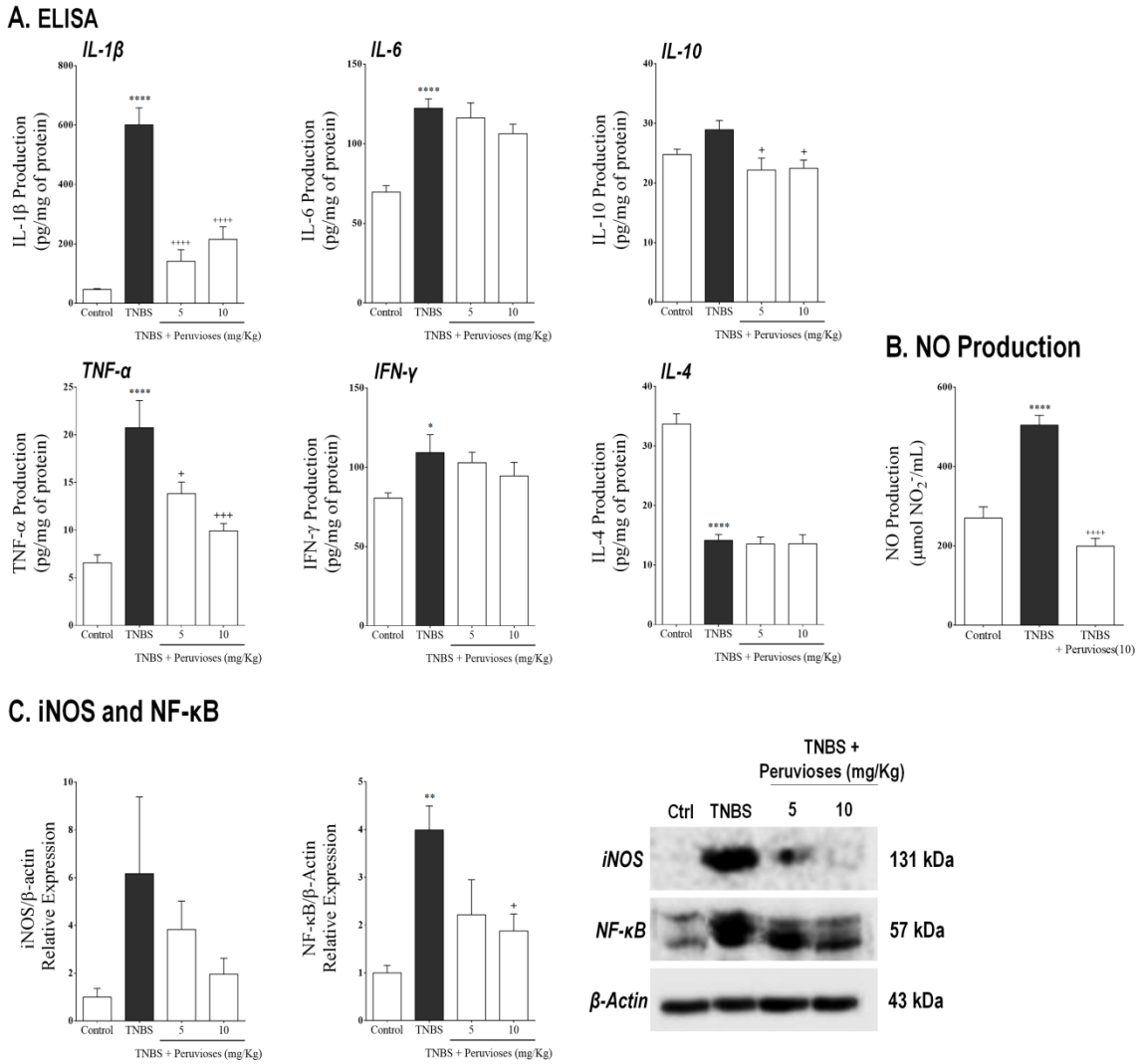


Figure 5.5 Peruvioses A and B reduce NO and cytokines production probably by inhibition of NF-κB pathway. (A) Cytokines (IL-1β, IL-6, IL-10, TNF-α, IFN-γ, and IL-4) levels were measured by ELISA. **(B)** NO production was quantified employing Griess reaction. **(C)** Expression of iNOS (cytoplasmic) and NF-κB (nuclear) were evaluated by immunoblotting. Results are representative of at least two independent experiments, and are expressed as the mean ± SEM (n=7-10 per group). (*) P<0.05, (**) P<0.01, (***) P<0.001, and (****) P<0.0001 vs. control; (+) P<0.05, (+++) P<0.001, and (++++) P<0.0001 vs. TNBS group.

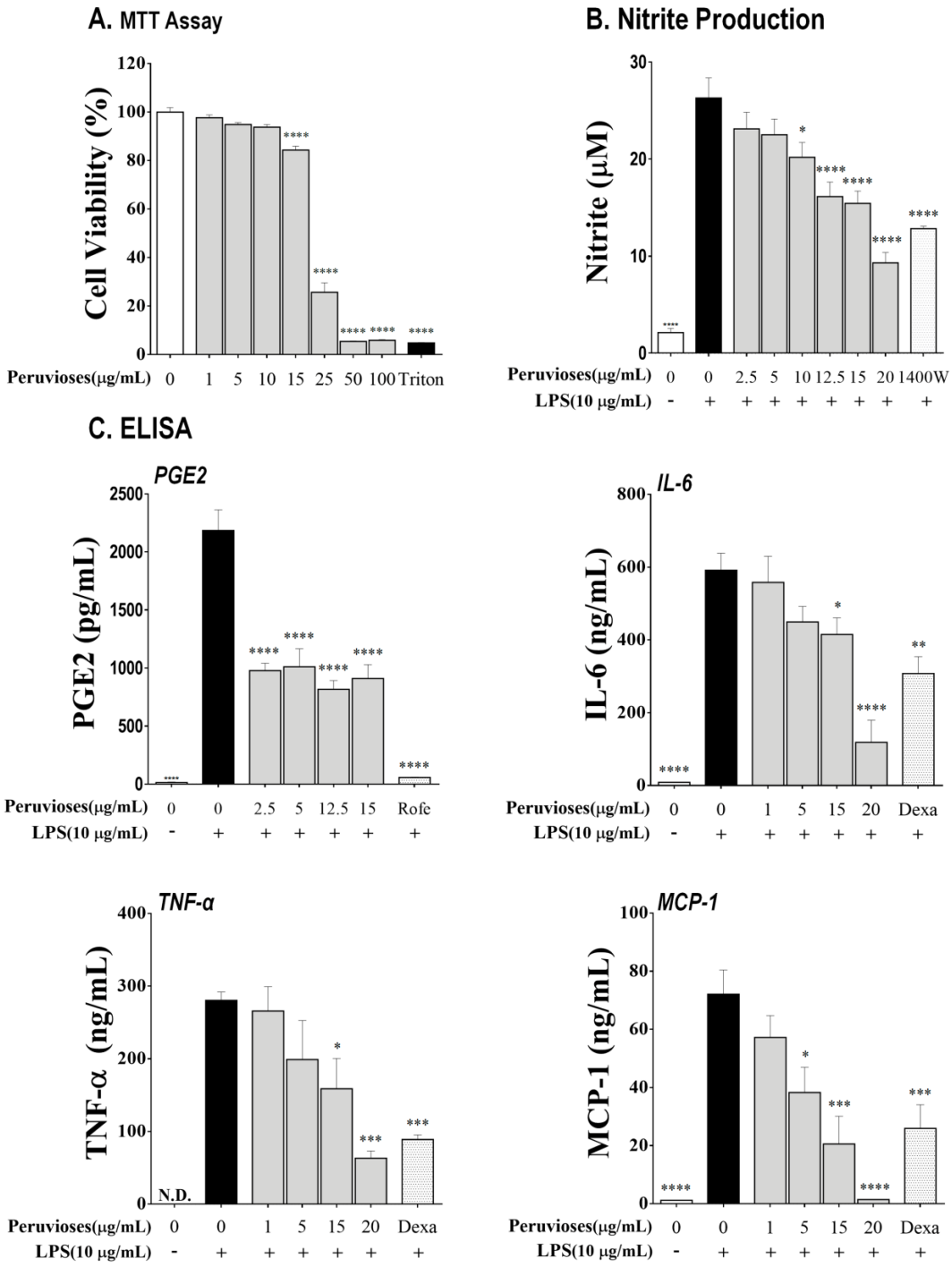


Figure 5.6. Peruvioses A and B inhibited pro-inflammatory mediators production by LPS-stimulated RAW 264.7 cells. Macrophages were cultured with peruvioses mixture (0-100 μg/mL) for 30 minutes and then stimulated with LPS (10 μg/mL) for 24 hours. **(A)** Cell viability was measured with the MTT assay, **(B)** Nitrite production was measured by Griess assay, **(C)** PGE2, **(D)** IL-6, **(E)** TNF-α, and **(F)** MCP-1 production were measured by ELISA. Results are representative of at least three independent experiments, and are expressed as the mean ± SEM (*) P<0.05, (**) P<0.01, (***) P<0.001, and (****) P<0.0001 vs. control.

5.4.4 Cytokine production is modulated by peruvioses A and B in macrophages activated with LPS.

In IBD patients, the proportion of lamina propria macrophages is increased, as well as the TLRs and NF-κB p65 expression, which is accompanied by an increased production of IL-1β, IL-6, and TNF-α.[31-34] To elucidate the potential beneficial effect of sucrose esters from *P. peruviana* on macrophages, we investigated the response of RAW 264.7 cells to lipopolysaccharide (LPS) activation in the presence or absence of tested compounds (15-1 μg/mL).

The mixture of peruviose A and B inhibited significantly the LPS-induced production of NO, PGE2, IL-6, TNF-α, and MCP-1, in a concentration-dependent manner (P<0.05), without affecting cell viability as demonstrated by MTT assay (Figure 5.6). Surprisingly, IL-1β production was not modified by tested compounds (data not shown).

To verify that the bioactivity of sucrose esters was not related to an antioxidant effect, we evaluated the scavenging activity of compounds on $NO^{\bullet}$, $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals in a non-cellular system. Sucrose esters from *P. peruviana* did not exert an important scavenging effect, showing inhibition lower than 3% at 100 μg/mL (data not shown).

5.5 Discussion

The mixture of peruviose A and B, sucrose esters isolated from *P. peruviana* calyces, has been recognized to be a promising anti-inflammatory agent.[18] Here, we show evidence of the beneficial effect of these sucrose esters in TNBS-induced colitis model. Overall, our results suggest that the amelioration of the altered immune response, that characterizes the colonic inflammatory process induced by TNBS, is involved in the favorable recovery of animals treated with these compounds.

The aerial surface of many genus of the Solanaceae family, such as *Datura*, *Lycopersicon*, *Nicotiana*, *Petunia*, *Physalis,* and *Solanum*, are covered by glandular trichomes, whose unique and versatile secretory metabolism contributes to the wide variety of secondary metabolites of these plants.[35, 36] Glucose and sucrose esters has been characterized as the main compounds present on the glandular trichome or its sticky exudate, which is believed to protect the plants and fruits from insect and infections.[37]

Previous studies have demonstrated that *Physalis* genus is an abundant source of sucrose esters.[38] So far, twenty sucrose esters have been isolated from *P. viscosa*, *P. nicandroides* var attenuata, *P. sordida*, *P. solanaceus, P. peruviana, P. neomexicana,* and *P. philadelphica.*[18, 39-44] Among the pharmacological activities reported for these secondary carbohydrates, their anti-inflammatory effect appears to be the most important.[38] Particularly, we have focused in the study of *P. peruviana* calyces, where sucrose esters are the most important metabolites.

In this study, administration of the mixture of peruviose A and B was carried by intraperitoneal route. The noxious response (writhing and piloerection) that occurred after injection of test compounds (20 mg/Kg/day) was not unexpected, since we previously found this effect when treating mice at higher doses.[18] However, the latency and magnitude of discomfort after administration with peruvioses A and B at 20 mg/Kg, was significantly less severe than the previously studied dosages (>100 mg/Kg), where acute toxicological effects of sucrose esters resulted in pronounced pain, piloerection, and hypoactivity.[18] This evidence indicates that sucrose esters produce local nociceptive effects in rodents.

In contrast, the 15-days administration of sucrose ester (5 and 10 mg/Kg) to Wistar rats showed no evidence of nociception/toxicity attributable to test compounds. Indeed, the histological examination revealed no changes on any of the animals treated with peruvioses when compared to control group. Nevertheless, pain-related behavioral responses to intraperitoneal administration of sucrose esters remains surprising, since carbohydrate polyesters, especially esters of sucrose and glucose, are commonly employed surfactants, that have been approved as food, cosmetics, and pharmaceutical additives based on safety studies on animals and humans.[45, 46]

In fact, sucrose esters have been proposed as emulsifiers, stabilizers, and absorption/penetration enhancers in drug delivery systems, especially for parenteral[47], nasal[48, 49], or topical administration[50]. Therefore, our data encourage further studies on their toxicological effect when administration routes different to oral gavage are employed. In the case of peruvioses mixture, additional research including longer periods of administration, inclusion of both male and female animals, and assessment of its mutagenic and carcinogenic potential, should be performed in order to support their safety.

To evaluate the effect of the mixture of peruviose A and B on intestinal inflammation, we employed the TNBS-induced colitis model. Among chemically induced models, TNBS remains as one of the most commonly employed, since it is considered a useful tool to provide proof of concept for therapeutic interventions in a simple, time saving, and relatively inexpensive setting.[51] Interestingly, many studies regarding complementary and alternative medicine on IBD have been done using this model.[52]

Although first developed for use in rats [23], this model has become widely used for inducing colitis in zebrafish [53, 54], mice [55], rabbits [56], pigs [57], and dogs [58]. TNBS is a hapten administered as an enema in combination with ethanol (30–50%), to break the mucosal barrier and allow TNBS penetration into the bowel wall.[59] While some of the colonic damage is attributable to the caustic properties of the TNBS/ethanol solution,[60] inflammation is mainly induced by haptenization of host proteins, which

provokes severe ulcerations of the mucosal characterized by transmural infiltration of mononuclear cells.[61, 62] Results of this study demonstrated that peruviose A and B from *P. peruviana* were useful to ameliorate the damage induced in the colon of rats treated with TNBS either acutely (preventive set-up) or in an established disease (therapeutic set-up). In both settings, sucrose esters were able to improve the mucosal damage at macroscopic and histologic levels. However, the greatest beneficial effect of peruvioses was noticed in the therapeutic set-up (two weeks treatment), which indicates that a sufficient time of treatment is needed for sucrose esters to fully exert their effects.

Based on the time course of macroscopic, histologic and immunological changes, inflammation by TNBS could be divided in several phases. The acute application of the TNBS-colitis model, where damage is assessed 3 days after TNBS instillation, is characterized by intense tissue injury and a non-specific inflammatory response.[30] In comparison, one week after TNBS administration, an established/chronic phase of inflammation sets in. In this phase the inflammation is characterized by a wide distribution of ulcers, cell infiltration that leads to granuloma formation, and Crohn's disease-like cytokine profile that reach a maximum at two weeks.[63] In this sense, our data suggests the potential of sucrose esters from *P. peruviana* calyces to treat the chronic and persistent inflammation that characterizes IBD.

In fact, in therapeutic set-up experiments, we observed that test compounds significantly accelerated the recovery of TNBS-treated rats, reducing the mortality rate and weight loss, which was accompanied by reduced colon weight/length ratio, ulcer index and damaged area extension. With regard to the histological analysis, our findings were in agreement with the previously reported changes induced by TNBS administration.[23] Furthermore, sucrose esters demonstrated a remarkable reduction of histological damage, consistent with the macroscopic observations.

Previous studies have established that TNBS model shares many molecular features with human IBD, such as overproduction of IFN-γ mediated by T helper 1 (Th1) T cells.[64] Although TNBS-induced cytokine secretion patterns are by far more studied in mice, some studies have also verified a time-dependent changing pattern of Th1 cytokines in the rat model of TNBS, as well as important concordances with IBD transcriptomes.[63, 65] Consistently, we report that TNBS-treated rats exhibited a remarkable fold increase in iNOS, COX-2, IL-1β, IL-6, IL-10, and IL-17A expression, two weeks after TNBS instillation. ELISA analysis also demonstrated increased IFN-γ and TNF-α production, whereas IL-4 was diminished. Alternatively, the expression of markers of mucus/epithelial integrity (MUC-2, MUC-3, and TFF-3), were significantly impaired.

Peruvioses A and B from *P. peruviana* were able to partially restore the inflammatory mediators and cytokine imbalance, with marked diminution of IL-1β, TNF-α, and iNOS at

mRNA or protein levels. Moreover, sucrose esters normalized the expression of MUC-2 and TFF-3, which are secreted by goblet cells.[66] Accordingly, we observed a higher number of goblet cells as evidenced by PAS-staining, which suggests that treatment with peruvioses promoted epithelial regeneration in TNBS-treated rats.

Neutrophils recruitment to the intestine is a critical component of the inflammatory response. This process relies on selectins, integrins, and adhesion molecules both for transendothelial and epithelial transmigration.[67] The association of the elevated expression of these molecules and intestinal inflammation has been well documented in humans and animals models of IBD.[67] For instance, the up-regulation of E-selectin (endothelial-selectin; CD62E); P-selectin (Platelet-selectin; CD62P); intercellular adhesion molecule 1 (ICAM-1); and vascular cell adhesion protein 1 (VCAM-1)] has been reported during TNBS-induced colitis in rats, peaking two weeks after induction of disease.[63, 68, 69]. Indeed, it is known that their expression is regulated at the transcriptional level by TNF-α and IL-1β.[68, 70, 71] In agreement, our data showed that TNBS provoked an abnormal recruitment of inflammatory cells to the colon tissue. Certainly, it is well established that this inflammatory infiltrate, mainly composed by neutrophils, is important for the development of colon edema.[72] From our point of view, the strong reduction of TNF-α and IL-1β, both at the transcriptional and protein levels, caused by the sucrose esters from *P. peruviana* might be correlated to the significant reduction of MPO activity, which has been shown to be proportional to neutrophils infiltration in the colonic tissue.[73]

Infiltrating macrophages play a crucial role in the pathogenesis of TNBS-induced colitis.[74] To clarify whether sucrose esters from *P. peruviana* were able to attenuate cytokine imbalance in macrophages, we employed LPS-stimulated RAW 264.7 cells. In the presence of peruvioses mixture, LPS-stimulated macrophages produced less NO, PGE2, IL-6, TNF-α, and MCP-1, at non-toxic concentrations.

Alternatively, sucrose esters effects were not related to antioxidant properties, as demonstrated using DPPH and ABTS assays. Although the anti-inflammatory effect of plant derived sucrose esters have been previously described, research has focused on COX-2 (PGE2) and iNOS (NO) inhibition [18, 44, 75], our results demonstrated that sucrose esters are able to modulate inflammation with a more complex mechanism of action. Nevertheless, since all the genes modulated by peruvioses A and B mixture, either *in vivo* or *in vitro*, are gene targets of the transcription factor NF-κB, we propose that the immunomodulatory effects of sucrose esters might result from inactivation of NF-κB signal pathway. Indeed, this study demonstrated that peruvioses inhibited the mRNA expression and the nuclear translocation of NF-κB in rats treated with TNBS.

Although the experimental results from the TNBS-colitis model demonstrated the beneficial effect of sucrose esters from *P. peruviana*, there are several caveats that worth

discussing. First, we only demonstrated the efficacy of peruvioses A and B to treat colonic inflammation in one animal model, which does not fully resemble IBD pathology in humans. Thus, the bioactivity of test compounds should be determined in at least another model. Additionally, TNBS-induced colitis, as applied in this work, is more representative of an acute IBD flare-up than a chronic condition; therefore future studies should include an experimental setting of chronic inflammation to determine whether sucrose esters are useful as long-term therapy for IBD.[76] On the other hand, given the chronic nature of IBD, administration of test compound per oral should be preferred [61]; however, we employed intraperitoneal administration of peruvioses. Although debatable, this route of administration was chosen given the fact that sucrose esters are not absorbed but metabolized in the gastrointestinal tract [77], thus their bioavailability could be limited.

In summary, our results demonstrate that sucrose esters of *P. peruviana* calyces ameliorates the symptoms and progression of colitis in the TNBS-induced model by improving the epithelial recovery and modulating the cytokine unbalance associated with colitis, probably through suppression of NF-κB activation. Thus, sucrose esters from *P. peruviana* might be an interesting new complementary herbal alternative to treat IBD.

5.6 References

1. Burisch, J. and P. Munkholm, *Inflammatory bowel disease epidemiology.* Current Opinion in Gastroenterology, 2013. **29**(4): p. 357-362.
2. Wong, S.H. and S.C. Ng, *What can we learn from inflammatory bowel disease in developing countries?* Curr Gastroenterol Rep, 2013. **15**(3): p. 313.
3. Ford, A.C., et al., *Efficacy of Biological Therapies in Inflammatory Bowel Disease: Systematic Review and Meta-Analysis.* American Journal of Gastroenterology, 2011. **106**(4): p. 644-659.
4. Cosnes, J., et al., *Epidemiology and natural history of inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1785-94.
5. Li, R., et al., *An old herbal medicine with a potentially new therapeutic application in inflammatory bowel disease.* Int J Clin Exp Med, 2011. **4**(4): p. 309-19.
6. Bertomoro, P., et al., *Regional variations in the use of complementary and alternative medicines (CAM) for inflammatory bowel disease patients in Italy: an IG-IBD study.* J Crohns Colitis, 2010. **4**(3): p. 291-300.
7. Rogler, G., et al., *Role of biological therapy for inflammatory bowel disease in developing countries.* Gut, 2012. **61**(5): p. 706-12.
8. Cragg, G.M. and D.J. Newman, *Natural products: a continuing source of novel drug leads.* Biochim Biophys Acta, 2013. **1830**(6): p. 3670-95.
9. Hur, S.J., et al., *Review of natural products actions on cytokines in inflammatory bowel disease.* Nutr Res, 2012. **32**(11): p. 801-16.
10. Rahimi, R., S. Mozaffari, and M. Abdollahi, *On the use of herbal medicines in management of inflammatory bowel diseases: a systematic review of animal and human studies.* Dig Dis Sci, 2009. **54**(3): p. 471-80.
11. Rahimi, R., S. Nikfar, and M. Abdollahi, *Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: a meta-analysis.* World J Gastroenterol, 2013. **19**(34): p. 5738-49.

12. Langhorst, J., et al., *Systematic review of complementary and alternative medicine treatments in inflammatory bowel diseases.* J Crohns Colitis, 2015. **9**(1): p. 86-106.
13. Simbaqueba, J., et al., *Development and characterization of microsatellite markers for the Cape gooseberry Physalis peruviana.* PLoS One, 2011. **6**(10): p. e26719.
14. Fischer, G. and D. Miranda, *Uchuva (Physalis peruviana L.)*. Manual para el cultivo de frutales en el trópico2012: Produmedios. 851-853.
15. Bravo, K., et al., *Influence of cultivar and ripening time on bioactive compounds and antioxidant properties in Cape gooseberry (Physalis peruviana L.).* J Sci Food Agric, 2015. **95**(7): p. 1562-9.
16. Fischer, G., P.J. Almanza-Merchán, and D. Miranda, *Importancia y cultivo de la uchuva (Physalis peruviana L.).* Revista Brasileira de Fruticultura, 2014. **36**(1): p. 01-15.
17. Fisher, G. and P. Lüdders, *Developmental changes of carbohydrates in Cape Gooseberry (Physalis peruviana L.) fruits in relation to the calyx and the leaves.* Agronomia Colombiana, 1997. **14**(2): p. 95-107.
18. Franco, L.A., et al., *Sucrose esters from Physalis peruviana calyces with anti-inflammatory activity.* Planta Med, 2014. **80**(17): p. 1605-14.
19. Mayorga, H., et al., *Glycosidically bound flavor compounds of cape gooseberry (Physalis peruviana L.).* J Agric Food Chem, 2001. **49**(4): p. 1904-8.
20. Ramadan, M.F. and J.T. Morsel, *Oil goldenberry (Physalis peruviana L.).* J Agric Food Chem, 2003. **51**(4): p. 969-74.
21. Castro, J., Y. Ocampo, and L. Franco, *Cape Gooseberry [Physalis peruviana L.] Calyces Ameliorate TNBS Acid-induced Colitis in Rats.* J Crohns Colitis, 2015. **9**(11): p. 1004-15.
22. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica, 2007. **27**(1): p. 110-5.
23. Morris, G.P., et al., *Hapten-induced model of chronic inflammation and ulceration in the rat colon.* Gastroenterology, 1989. **96**(3): p. 795-803.
24. Bobin-Dubigeon, C., et al., *Effects of tumour necrosis factor-alpha synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis.* Eur J Pharmacol, 2001. **431**(1): p. 103-10.
25. Arribas, B., et al., *Di-D-fructose dianhydride-enriched caramels: effect on colon microbiota, inflammation, and tissue damage in trinitrobenzenesulfonic acid-induced colitic rats.* J Agric Food Chem, 2010. **58**(10): p. 6476-84.
26. Castro, J.P., Y.C. Ocampo, and L.A. Franco, *In vivo and in vitro anti-inflammatory activity of Cryptostegia grandiflora Roxb. ex R. Br. leaves.* Biological research, 2014. **47**(1): p. 32.
27. Garrido, G., et al., *In vivo and in vitro anti-inflammatory activity of Mangifera indica L. extract (VIMANG®).* Pharmacological research, 2004. **50**(2): p. 143-149.
28. Ferrari, M., M.C. Fornasiero, and A.M. Isetta, *MTT colorimetric assay for testing macrophage cytotoxic activity in vitro.* J Immunol Methods, 1990. **131**(2): p. 165-72.
29. Stuehr, D.J. and C.F. Nathan, *Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells.* J Exp Med, 1989. **169**(5): p. 1543-55.
30. te Velde, A.A., M.I. Verstege, and D.W. Hommes, *Critical appraisal of the current practice in murine TNBS-induced colitis.* Inflamm Bowel Dis, 2006. **12**(10): p. 995-9.
31. Kusugami, K., et al., *Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent.* Dig Dis Sci, 1995. **40**(5): p. 949-59.
32. Hausmann, M., et al., *Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation.* Gastroenterology, 2002. **122**(7): p. 1987-2000.
33. Neurath, M.F., et al., *Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease.* Ann N Y Acad Sci, 1998. **859**: p. 149-59.

34. Reinecker, H.C., et al., *Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease.* Clin Exp Immunol, 1993. **94**(1): p. 174-81.
35. King, R.R., et al., *Characterization of 2,3,4,3'-tetra-O-acylated sucrose esters associated with the glandular trichomes of Lycopersicon typicum.* J Agric Food Chem, 1993. **41**(3): p. 469-473.
36. Shapiro, J.A., J.C. Steffens, and M.A. Mutschler, *Acylsugars of the wild tomato Lycopersicon pennellii in relation to geographic distribution of the species.* Biochemical Systematics and Ecology, 1994. **22**(6): p. 545-561.
37. Eich, E., *Secondary Metabolites Derived from Fatty Acids and Carbohydrates*, in *Solanaceae and Convolvulaceae: Secondary Metabolites: Biosynthesis, Chemotaxonomy, Biological and Economic Significance (A Handbook)*2008, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 525-582.
38. Zhang, W.N. and W.Y. Tong, *Chemical Constituents and Biological Activities of Plants from the Genus Physalis.* Chem Biodivers, 2016. **13**(1): p. 48-65.
39. Ovenden, S.P., et al., *Physaloside A, an acylated sucrose ester from Physalis viscosa.* J Nat Prod, 2005. **68**(2): p. 282-4.
40. Maldonado, E., et al., *Sucrose esters from the fruits of Physalis nicandroides var. attenuata.* J Nat Prod, 2006. **69**(10): p. 1511-3.
41. Perez-Castorena, A.L., M. Martinez, and E. Maldonado, *Labdanes and sucrose esters from Physalis sordida.* J Nat Prod, 2010. **73**(7): p. 1271-6.
42. Perez-Castorena, A.L., et al., *New sucrose esters from the fruits of Physalis solanaceus.* Carbohydr Res, 2012. **352**: p. 211-4.
43. Cao, C.M., et al., *Withanolides and Sucrose Esters from Physalis neomexicana.* J Nat Prod, 2015. **78**(10): p. 2488-93.
44. Zhang, C.R., et al., *New antiinflammatory sucrose esters in the natural sticky coating of tomatillo (Physalis philadelphica), an important culinary fruit.* Food Chem, 2016. **196**: p. 726-32.
45. Szuts, A. and P. Szabo-Revesz, *Sucrose esters as natural surfactants in drug delivery systems - a mini-review.* Int J Pharm, 2012. **433**(1-2): p. 1-9.
46. Zheng, Y., et al., *Suggar Fatty Acid Esters*, in *Polar Lipids: Biology, Chemistry, and Technology*, M.U. Ahmad and X. Xu, Editors. 2015, Elsevier. p. 215-273.
47. Youan, B.B., A. Hussain, and N.T. Nguyen, *Evaluation of sucrose esters as alternative surfactants in microencapsulation of proteins by the solvent evaporation method.* AAPS PharmSci, 2003. **5**(2): p. E22.
48. Kurti, L., et al., *The effect of sucrose esters on a culture model of the nasal barrier.* Toxicol In Vitro, 2012. **26**(3): p. 445-54.
49. Ahsan, F., et al., *Sucrose cocoate, a component of cosmetic preparations, enhances nasal and ocular peptide absorption.* Int J Pharm, 2003. **251**(1-2): p. 195-203.
50. Nava-Arzaluz, M.G., E. Piñón-Segundo, and A. Ganem-Rondero, *Sucrose Esters as Transdermal Permeation Enhancers*, in *Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement: Modification of the Stratum Corneum*, N. Dragicevic and I.H. Maibach, Editors. 2015, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 273-290.
51. Pizarro, T.T., et al., *Mouse models for the study of Crohn's disease.* Trends Mol Med, 2003. **9**(5): p. 218-22.
52. Mizoguchi, A., *Animal models of inflammatory bowel disease.* Prog Mol Biol Transl Sci, 2012. **105**: p. 263-320.
53. Fleming, A., J. Jankowski, and P. Goldsmith, *In vivo analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study.* Inflamm Bowel Dis, 2010. **16**(7): p. 1162-72.

54. Oehlers, S.H., et al., *A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents.* Dev Dyn, 2011. **240**(1): p. 288-98.
55. Neurath, M.F., et al., *Antibodies to interleukin 12 abrogate established experimental colitis in mice.* J Exp Med, 1995. **182**(5): p. 1281-90.
56. Percy, W.H., et al., *In vitro changes in the properties of rabbit colonic muscularis mucosae in colitis.* Gastroenterology, 1993. **104**(2): p. 369-76.
57. Merritt, A.M., C.D. Buergelt, and L.C. Sanchez, *Porcine ileitis model induced by TNBS-ethanol instillation.* Dig Dis Sci, 2002. **47**(4): p. 879-85.
58. Shibata, Y., M. Taruishi, and T. Ashida, *Experimental ileitis in dogs and colitis in rats with trinitrobenzene sulfonic acid - colonoscopic and histopathologic studies.* Gastroenterol Jpn, 1993. **28**(4): p. 518-27.
59. Wirtz, S. and M.F. Neurath, *Animal models of intestinal inflammation: new insights into the molecular pathogenesis and immunotherapy of inflammatory bowel disease.* Int J Colorectal Dis, 2000. **15**(3): p. 144-60.
60. Wallace, J.L., et al., *Hapten-induced chronic colitis in the rat: alternatives to trinitrobenzene sulfonic acid.* J Pharmacol Toxicol Methods, 1995. **33**(4): p. 237-9.
61. Dothel, G., et al., *Animal models of chemically induced intestinal inflammation: predictivity and ethical issues.* Pharmacol Ther, 2013. **139**(1): p. 71-86.
62. Morampudi, V., et al., *DNBS/TNBS colitis models: providing insights into inflammatory bowel disease and effects of dietary fat.* J Vis Exp, 2014(84): p. e51297.
63. Sun, F.F., et al., *Pattern of cytokine and adhesion molecule mRNA in hapten-induced relapsing colon inflammation in the rat.* Inflammation, 2001. **25**(1): p. 33-45.
64. Neurath, M., I. Fuss, and W. Strober, *TNBS-colitis.* Int Rev Immunol, 2000. **19**(1): p. 51-62.
65. Brenna, O., et al., *Relevance of TNBS-colitis in rats: a methodological study with endoscopic, histologic and transcriptomic characterization and correlation to IBD.* PLoS One, 2013. **8**(1): p. e54543.
66. Johansson, M.E., *Mucus layers in inflammatory bowel disease.* Inflamm Bowel Dis, 2014. **20**(11): p. 2124-31.
67. Fournier, B. and C. Parkos, *The role of neutrophils during intestinal inflammation.* Mucosal immunology, 2012. **5**(4): p. 354-366.
68. Sans, M., et al., *VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis.* Gastroenterology, 1999. **116**(4): p. 874-83.
69. Rojas-Cartagena, C., I. Flores, and C.B. Appleyard, *Role of tumor necrosis factor receptors in an animal model of acute colitis.* Cytokine, 2005. **32**(2): p. 85-93.
70. Wright, R.D. and D. Cooper, *Glycobiology of leukocyte trafficking in inflammation.* Glycobiology, 2014. **24**(12): p. 1242-51.
71. Bevilacqua, M., et al., *Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins.* Science, 1989. **243**(4895): p. 1160-1165.
72. Buell, M.G. and M.C. Berin, *Neutrophil-independence of the initiation of colonic injury. Comparison of results from three models of experimental colitis in the rat.* Dig Dis Sci, 1994. **39**(12): p. 2575-88.
73. Krawisz, J.E., P. Sharon, and W.F. Stenson, *Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models.* Gastroenterology, 1984. **87**(6): p. 1344-50.
74. Kanai, T., et al., *Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease.* Gastroenterology, 2001. **121**(4): p. 875-88.
75. Wang, N., et al., *Bioactive sucrose esters from Bidens parviflora.* Phytochemistry, 2003. **62**(5): p. 741-6.
76. Dudley, J.T., et al., *Computational repositioning of the anticonvulsant topiramate for inflammatory bowel disease.* Sci Transl Med, 2011. **3**(96): p. 96ra76.

77. Kiss, L., et al., *Sucrose esters increase drug penetration, but do not inhibit p-glycoprotein in Caco-2 intestinal epithelial cells.* J Pharm Sci, 2014. **103**(10): p. 3107-19.

6 CHAPTER SIX. Safety of sucrose esters from *Physalis peruviana* L. in a 28-day repeated-dose study using CD-1 (ICR) mice.

Yanet C. Ocampo[1], Daneiva C. Caro[1], David E. Rivera[1], Luis A. Franco[1]

[1]Biological Evaluation of Promising Substances Group, Faculty of Pharmaceutical Sciences, University of Cartagena, Cartagena, Colombia.

6.1 Abstract

Peruvioses A and B isolated from *Physalis peruviana* L. calyces have proved to be effective anti-inflammatory and immunomodulatory compounds. This study aimed to investigate their sub-acute toxicity and genotoxicity. For this, CD-1(ICR) mice were treated intraperitoneally with peruvioses at doses of 2.5, 5, and 10 mg/Kg/day for 28 consecutive days, to simulate therapeutic and over-therapeutic dosage levels. At the end of the treatment, animals were sacrificed and their organs weighted, and blood and tissue samples were collected. Toxicological endpoints included clinical signs; food consumption; body and organ weights; hematological and biochemical parameters; as well as macroscopic and microscopic examination of tissues. The results showed no significant differences between treated animals and control group at macroscopic, histological, molecular, and biochemical levels. In addition, a combination of mammalian erythrocyte, comet assay in peripheral blood cells, and Ames test, did not reveal genotoxic effects induced by peruvioses. Taken together, our data suggests that peruvioses A and B can be safely employed to treat inflammatory diseases.

Keywords: Sucrose esters, *Physalis*, Solanaceae, Toxicity.

6.2 Introduction

Physalis peruviana L. is an herbaceous, semi-shrub, upright perennial plant, belonging to Solanaceae family.[1] Although native to South American Andes, *P. peruviana* has been widely introduced around the world reaching importance in countries as South Africa, Kenya, Zimbabwe, Australia, New Zealand, Hawaii, India, Malaysia, and China.[2] In Colombia, *P. peruviana* is one of the most important exotic fruit crops, representing a major source of profit for some regions of the country.[3, 4] With a production of 10.771 tons in 2011, and an export demand of 50%, Colombia is positioned as the largest exporter of this fruit in the world.[2, 5]

In addition to the economical and nutritional importance of *P. peruviana,* the fruit as well as the whole plant material is employed in folk medicine with a variety of purposes.[1] Specifically, leaves and small stems along with the calyces, a bladder-like accrescent

structure that encloses the mature fruit [6]; are widely employed as traditional herbal remedy to relieve fever, edema and inflammation.[7] Current research on *P. peruviana* calyces has focused on verifying the bioactivity of its total ether extract using animal models of inflammation, including TPA-induced ear edema and TNBS-induced colitis.[7-10] Previous chemical investigations have established sucrose esters as the major secondary metabolites of this organ. The mixture of two sucrose esters, peruvioses A and B, proved to significantly reduce the inflammation induced by λ-carrageenan in a dosage-dependent manner, probably due to an inhibition of nitric oxide and prostaglandin E2.[11]

Considering that sucrose esters have been isolated from aerial parts, flower, fruits, and calyces from seven *Physalis* species [12], presumably their presence might be related to the anti-inflammatory activity described for some plants of this genus.[13] Indeed, the therapeutic potential of sucrose esters to treat inflammation has been well described. For instance, it was reported that physordinoses A-D, from *P. sordida*, exhibited a remarkable potential to diminish TPA-induced edema with similar activity to indomethacin.[13] Recently, [14] found that five sucrose esters isolated from *P. philadelphica* inhibited cyclooxygenase (COX)-1 and COX-2. In addition, sucrose esters are recognized for its prominent antimicrobial activity. Although variable results have been reported regarding the sensitivity of bacterial strains, mechanism of action, and structure/activity relationship [15, 16], the potential of sucrose esters as multifunctional metabolites is clear, since they might be effective to treat infections and inflammatory conditions.

Besides the increasing interest in sucrose esters as a therapeutic herbal derived agent, there is a widespread current use of these compounds as surfactants; particularly in food, cosmetic and pharmaceutical industries.[17] They are highly valued because they are tasteless, odorless, non-toxic, non-irritant, provide good stabilizing and conditioning properties, and are produced from sustainable and renewable resources: nonpolar fatty acids and sucrose.[18] Regarding their legal status, sucrose esters have broad application in the food industry without any safety or regulations issues.[15] The European Union (Directive No 95/2/EC), the United States of America (Title 21 of the CFR § 172.860 and § 172.869), Canada (IMA Canada Gazette 14-02-2004), and Japan, have approved their employment in general foodstuffs [18]. This indicates the general safety of these surfactants through oral ingestion, which is well supported by toxicological, carcinogenic and absorption/metabolic studies in mammals and humans.[15] In the case of pharmaceutical and cosmetic applications, sucrose esters have also been proposed as emulsifiers, stabilizers, and absorption/penetration enhancers, especially for parenteral [19], nasal [20, 21], or topical administration.[17] Nevertheless, there is a lack of toxicological studies of sucrose esters employed by parenteral routes of administration.

In nature, sucrose esters are mainly found as mono- to hexa-esterified compounds, with acyl groups varying in their length (from C2 to C12) and backbone structure (iso-branched,

anteiso-branched and straight carbon chain).[22] This diversity in chemical structure provides them with differences in physicochemical properties and bioactivity. For instance, sugar esters can be classified as bitter and non-bitter depending on the length of the carbon chain of the acyl group [14], similarly they might be repellent to predators or attractors to pollinators.[23] In this context, the toxic effects of naturally derived sucrose esters might be different than those from their synthetic analogs.

On the other hand, as important pharmacologically active sucrose esters have been isolated from *Physalis* species, the examination of their toxicological effect is necessary. In order to continue our work on *P. peruviana* calyces and determine its possible application to develop new anti-inflammatory drugs, we conducted an evaluation of the safety of peruvioses A and B using a 28-days toxicity study design in CD-1 (ICR) mice. In addition, a battery of genotoxicity tests was carried out to further evaluate the carcinogenic potential of compounds.

6.3 Materials and Methods.

6.3.1 Plant Material, Sucrose Esters isolation and preparation.

Physalis peruviana L. was collected in La Mesa, Colombia. A voucher specimen (COL-512200) has been deposited at Herbario Nacional Colombiano (Instituto de Ciencias Naturales, Universidad Nacional de Colombia), Bogotá-Colombia. Isolation of Peruviose A and B from the calyces of *P. peruviana* was carried out as previously described.[11] Purity of sucrose esters mixture was estimated to be 99.05% by HPLC-MS (LaChrom Elite®, Merck-Hitachi, Darmstadt, Germany) and their structures were confirmed by comparison of their IR (Perkin Elmer 1600 series FT-IR spectrometer, Norwalk, CT, USA), and ^{1}H NMR data (Bruker spectrometer, 300 MHz, Billerica, MA, USA) with those previously reported [11]. Given the low solubility of the mixture of peruviose A and B from *P. peruvia*na in water, sucrose esters were co-precipitated with polyvinylpyrrolidone (PVP K-30, USP grade) in a 1:4 (w/w) ratio by solvent evaporation technique.

6.3.2 Animals

Male and female CD-1 (ICR) mice, approximately 4-weeks of age, were provided in good health by Instituto Nacional de Salud (Bogotá, Colombia). Animals were allowed to acclimatize for two weeks before use with food and water available *ad libitum*. Mice were housed in groups of five mice in filtered-capped polycarbonate cages (32 cm long × 20 cm wide × 21 cm high), containing wood shavings and maintained in a controlled environment with temperature at 20.14±0.11 °C, humidity between 41 to 53 %, under 12 h light/darkness cycle (6:00 am to 6:00 pm). Individual body weights were recorded every other day during the acclimation/pretest period to ensure the use of healthy animals.

This study was carried out in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU). Experiments followed a protocol approved by the Committee of Ethics in Research of the University of Cartagena (Minutes of October 14, 2010). All efforts were made to minimize animal suffering.

6.3.3 Animal Treatment and Dosing

The safety of the mixture of peruvioses A and B was evaluated in a 28-day repeated-dose study (Figure 6.1A), that was designed and conducted in accordance with the Guidelines for the Testing of Chemicals - Organization for Economic Cooperation and Development (OECD).[24] For this, CD-1 (ICR) mice were randomly assigned to groups of ten animals (five of each sex per group), at least one week prior to the start of exposure. Body weight values ranged from 22-28 g (mean 24.71±0.20 g) for the females, and 27-34 g (mean 29.88±0.26 g) for the males.

Peruvioses A and B co-precipitated with PVP-K30 was dissolved in sterile phosphate saline buffer (PBS, pH 7.3), prior to animal treatment, and administered intraperitoneally (ip) at three dosages levels (2.5, 5, and 10 mg/Kg/day). The highest dose was selected in reference to previous pharmacological studies in rats and acute toxicity evaluation in mice.[11, 25] Mitomycin C (MMC, Mixandex®, PiSA Pharmaceuticals, Mexico) was used at dosages of 0.5 mg/Kg/day ip, as control for *in vivo* micronucleus test [26]. MMC was dissolved in sterile water (Baxter International Inc., Colombia) and diluted in PBS before administration.

Test compounds were administered daily for a 28-day period. Mice from control group received PBS containing PVP-K30 only. Individual body weight, possible signs of toxicity, and food consumption per cage were monitored daily through the study. A detailed clinical examination was performed weekly following the five parameters of the Hippocratic screening (conscious state; activity and coordination of motor system and muscle toning; reflexes; activities on the central nervous system; and activities on the autonomic nervous system) by an investigator blinded to treatments.[27]

6.3.4 Urinalysis

Urine was collected on the last day of treatment (day 28), using the single animal method with clear plastic wrap as proposed by [28]. Changes in specific gravity (SG), pH, protein, nitrite, urobilinogen, bilirubin, glucose, and the presence of occult blood or ketones were assessed with urinary test papers (SD UroColor^{TM} 10, Standard Diagnostics, INC, Korea).

6.3.5 Gross Necropsy

Following the 28-day test period, all mice were fasted for 8-10 h, then anesthetized with 2-4% of sevoflurane (Sevorane®, Abbott, Vienna, Austria) using the open drop method.

Blood samples were obtained from the orbital venous plexus for subsequent analysis. Thereafter, detailed post-mortem necropsy was performed, including examination of the external surface, all orifices, as well as thoracic and abdominal cavities. Kidneys, spleen, heart, lungs, and testes/ovaries were weighed and fixed in 10%-buffered formalin for histological analysis. In the case of the liver, the entire organ (including the gall bladder) was weighed, and a part of the left lateral lobe was sliced and either fixed in 10%-buffered formalin or placed in RNA Later® (Qiagen, Valencia, USA) and stored at -80°C until the RNA extraction was performed.

6.3.6 Histology Analysis

Organs fixed in 10% buffered formalin were embedded in paraffin, sliced at 5 µm, and stained with hematoxylin and eosin (H&E) according to standard protocols. Samples were blindly analyzed by an experienced pathologist employing light microscopy (Olympus BX41, Tokyo, Japan). Full histopathology was carried out with all the organs by analyzing parameters like tissue degeneration, necrosis, apoptosis, inflammatory cell infiltration, congestion, and fibrosis. Images were captured at 10X and 20X magnifications on a Axio Lab.A1 microscope (Carl Zeiss, Oberkochen, Germany), coupled to a computer driven Zeiss AxioCAM digital camera (ICc5).

6.3.7 Blood biochemical profile

For biochemical analysis, blood samples were obtained in clean, no heparinized vials and allowed to clog for 30 minutes. Then samples were centrifuged at 1500 g for 10 min to obtain serum, which was stored at -80 °C until further analysis. Fasting serum levels of glucose, albumin, urea nitrogen, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were determined with an automatic blood chemical analyzer (A15 Random Access Analyzer, Biosystems, Spain). Blood samples were analyzed individually and not pooled.

6.3.8 Hematology analysis and peripheral blood micronucleus (MN) assay

Peripheral blood was collected as described above. Two blood smears were prepared for each blood sample, air dried, stained with Wright´s solution (Merck, Darmstadt, Germany), and labeled with a random number to avoid the identification of the treatment group during analysis. Coded slides were analyzed by two independent investigators using a Zeiss Axio Lab.A1 microscope (Carl Zeiss, Oberkochen, Germany). White blood cell differential counts (lymphocytes, reactive lymphocytes, immature lymphocytes, monocytes, neutrophils, eosinophil, and basophils) were performed by randomly examining 100 cells per slide at 100X magnification. In addition, for each slide (a) 1000 polychromatic and/or normochromatic erythrocytes (PCE and NCE, respectively) were examined and the PCE/NCE ratio was determined; (b) 1000 consecutive PCE were examined to report the incidence of micronucleated PCE (MN-PCE); and (C) 2000 consecutive NCE were examined to report the incidence of micronucleated NCE (MN-NCE).[29]

6.3.9 Bone marrow MN assay

MN assay using bone marrow was performed according to the protocol established by [30] and modified by [31]. Briefly, both femurs were excised intact from each animal and the bone marrow flushed out with fetal bovine serum (GIBCO-Invitrogen, Carlsbad, USA), and the cell suspension was centrifuged at 1000 rpm for 5 min. Bone marrow cells were re-suspended in a small volume of serum and 20 µL was smeared on two slides per animal. Slides were fixed in methanol, air-dried overnight, stained with Wright´s solution (Merck, Darmstadt, Germany), and labeled with a random number. Coded slides were analyzed by two researchers using a Zeiss Axio Lab.A1 microscope (Carl Zeiss, Oberkochen, Germany). For each slide, 200 PCE and/or NCE were examined and the PCE/NCE ratio was determined. In addition, 1000 PCE per slide were analyzed to determine the amount of MN-PCE.[29]

6.3.10 Neutral comet assay

The comet assay was performed in neutral conditions as described by Trevigen (Trevigen, Gaithersburg, USA) with some modifications.[32] Briefly, 6 µL of blood harvested from the orbital venous plexus was mixed with 75 µL of low-melting point agarose (Trevigen). Samples were spread on CometSlides^{TM} (Trevigen) and allowed to solidify. After lysis, slides were equilibrated and electrophoresis was performed at 300 mA for 1 h. Finally, DNA was precipitated, and slides air dried, fixed in ethanol, and stained with SYBR® Safe DNA stain (Invitrogen, Carlsbad, USA). Cells were then visualized using a fluorescent microscope (Axio Lab.A1, Carl Zeiss, Oberkochen, Germany). Representative pictures were recorded at 10X magnification with a Zeiss AxioCAM digital camera (ICc5).

To assess the extent and distribution of DNA damage a qualitative analysis was performed by three blinded scorers that examined 1000 randomly selected and non-overlapping comets per well (2000 comets/animal). For this, comets were classified visually according to tail size ranging from undamaged (class 0) to maximum DNA damage (class 4). DNA damage was expressed as the subtotal frequency of nucleoids (F2+3+4) with medium (class 2), high (class 3), and complete (class 4) damaged DNA.

For quantitative analysis, two scorers performed the comet analysis using Open Comet software.[33] The percentage of DNA in the comet tail (%Tail DNA), Olive Tail Moment (OTM) and Tail Moment were calculated from at least 1000 comets per well (2000 comets/animal) as a measurement of DNA damage.

6.3.11 Quantitative Real-time PCR (RT-PCR)

Total mRNA was isolated from liver tissue using the GeneJET™ RNA Purification kit (Thermo Fisher Scientific, Vilnius, Lithuania) as described by the manufacturer. RNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham,

USA). For each sample, 1.0 µg of RNA were employed as template for cDNA synthesis using Transcriptor Universal cDNA Master (Roche, Mannheim, Germany). Real-time PCR analysis was performed using the LightCycler® 96 System (Roche, Mannheim, Germany) with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Foster City, USA) and specific primers - Appendix 5 (Eurofins Genomics, Huntsville, USA) according to the manufacturer's instructions. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Duplicate cycle threshold (CT) values were analyzed by the comparative CT (ΔΔCT) method.

6.3.12 Ames Mutagenicity Test

Mutagenicity of sucrose esters from *P. peruviana* was assessed by using the commercial Muta-ChromoPlate™ Ames test kit (EBPI Inc., Ontario, Canada), which follows Ames´s test principle and uses *Salmonella typhimurium* (TA100). In brief, lyophilized bacteria were cultured overnight in nutrient broth at 37 °C. Peruvioses A and B were dissolved in ethanol, diluted in growth media to obtain the desired concentration (30 µg/mL), mixed with *S. typhimurium* inoculum, and incubated in both presence and absence of S9 fraction, for 3 days at 37 °C in sterile air-tight plastic bag. After incubation, microplates were examined visually: yellow, partially yellow or turbid wells were scored as positives and all purple wells were scored negative. Sodium azide and 2-aminoanthracene were employed as positive control and ethanol was employed as negative control.

6.3.13 Statistical Analysis

All values are expressed as mean ± standard error of the mean (S.E.M) for each group. One-way analysis of variance (ANOVA) followed by Dunnet´s *post hoc* test, was used to determine differences between treatment and the control group. In the case of comparisons between two groups, two tailed t-test, with Welch´s correction when appropriate, was employed. Values of $P<0.05$ were considered significant.

6.4 Results

6.4.1 Body weight and clinical score are not modified by treatment with sucrose esters from *Physalis peruviana*.

Changes in the body weight of animals from all experimental groups are shown in Figure 6. 1B. Both male and female mice treated with the mixture of peruvioses A and B (2.5, 5, and 10 mg/Kg/day, ip) gained weight without significant differences when compared to vehicle-treated animals. In contrast, the MMC-treated mice exhibited a statistically significant reduction in body weight compared with control group. There were no test article-related deaths suggesting the safety of the administration of sucrose esters from *P. peruviana*. When compared with the control group, animals treated with peruvioses A and B did not exhibited changes such as external appearance, behavior, locomotor activity, and changes in food consumption (data not shown). However, occasionally we observed that injection of

the highest dose (10 mg/Kg) elicited slight abdominal contractions and piloerection that disappeared 5-10 minutes after administration.

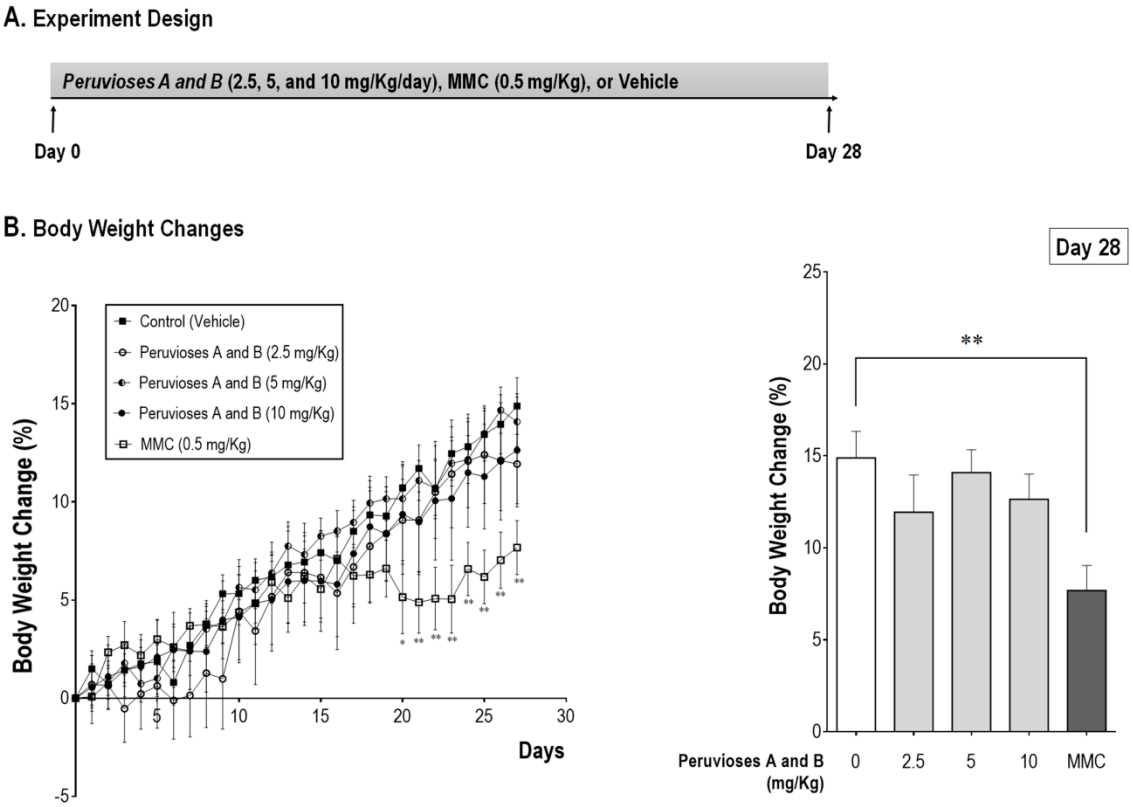


Figure 6.1. Body weight of mice is not affected by sucrose esters from *Physalis peruviana.* (A) Male and female CD-1 (ICR) mice were treated with the mixture of peruvioses (2.5, 5, and 10 mg/Kg/day, ip), mitomycin C (MMC, 0.5 mg/Kg/day, ip), or vehicle (PBS, pH 7.3) for a 28-day period. (B) Body weight was recorded daily and represented as percentage of weight change. Results are representative of two independent experiments, and are expressed as the mean ± S.E.M. (n=5-10 per group). (*) P<0.05 and (**) P<0.01 vs. control group.

6.4.2 Treatment with sucrose esters from *Physalis peruviana* does not alter vital organs at macroscopic or histological levels.

At the time of sacrifice, all animals treated with peruvioses A and B were in well condition, with normal physical appearance when compared to control group. In addition, absolute and relative weight of all the studied organs (liver, kidneys, spleen, heart, lungs and testes/ovaries) were found to be normal, regardless of sex or dosage level (Table 6.1). Pathological examination of the tissues on a gross basis did not revealed detectable abnormalities on mice treated with sucrose esters from *P. peruviana*. Consistently, histological evaluation did not show any microscopic disturbances on any of the studied organs related to the administration of peruvioses at the highest dose (Figure 6.2).

Table 6.1. Effect of sucrose esters from *Physalis peruviana* on absolute and relative organs weight of CD-1 (ICR) mice.

Treatment	Organ Weights (Relative Organ Weights)				
	Control (Vehicle)	Peruvioses A and B (mg/Kg/day)			MMC (0.5 mg/Kg/day)
		2.5	5	10	
Males *n*=5-10					
Body weight (g)	34.10±0.81	33.80±0.37	34.30±0.70	34.10±0.81	33.00±0.70**
Liver	2027.69±54.36 (5.90±0.13)	2125.65±173.96 (6.19±0.43)	2098.49±93.94 (6.10±0.23)	2125.44±121.66 (6.27±0.38)	2037.02±120.07 (6.21±0.43)
Kidneys	383.11±43.42 (1.09±0.11)	245.56±13.88 (0.72±0.04)	374.99±37.86 (1.10±0.11)	393.91±36.09 (1.17±0.10)	478.78±15.63 (1.46±0.06)
Spleen	164.23±6.61 (0.47±0.02)	160.46±14.41 (0.47±0.04)	162.31±9.91 (0.47±0.03)	168.62±15.74 (0.50±0.05)	128.34±14.30 (0.39±0.05)
Heart	190.29±6.88 (0.55±0.02)	165.18±8.28 (0.48±0.03)	191.44±6.16 (0.56±0.02)	183.33±9.43 (0.55±0.03)	186.14±11.04 (0.56±0.02)
Lungs	252.14±8.35 (0.73±0.02)	268.44±9.79 (0.79±0.03)	252.68±15.13 (0.74±0.04)	262.43±12.10 (0.78±0.03)	233.14±13.03 (0.71±0.05)
Testes	228.90±14.24 (0.66±0.04)	205.82±17.75 (0.60±0.05)	235.98±5.84 (0.69±0.02)	251.23±7.47 (0.74±0.03)	97.80±6.44**** (0.30±0.01)****
Females *n*=5-10					
Body weight (g)	27.90±0.69	27.50±0.43	28.10±0.67	26.80±0.33	26.00±0.32*
Liver	1471.04±64.95 (5.39±0.16)	1552.28±99.86 (5.54±0.35)	1552.33±42.73 (5.54±0.13)	1473.32±39.09 (5.54±0.14)	1410.26±25.26 (5.64±0.10)
Kidneys	245.38±30.19 (0.89±0.11)	176.97±4.97 (0.64±0.02)	247.78±30.50 (0.88±0.11)	234.29±27.35 (0.88±0.10)	286.10±11.29 (1.14±0.05)
Spleen	151.41±7.97 (0.55±0.03)	159.85±12.66 (0.57±0.04)	158.22±10.11 (0.57±0.04)	162.36±11.85 (0.61±0.04)	112.70±5.52* (0.45±0.02)
Heart	149.35±6.63 (0.54±0.02)	137.38±7.52 (0.49±0.03)	150.86±6.04 (0.54±0.02)	149.59±5.50 (0.56±0.02)	142.48±11.83 (0.57±0.02)
Lungs	233.13±7.31 (0.85±0.02)	239.50±19.66 (0.86±0.06)	229.74±9.51 (0.82±0.03)	229.50±9.01 (0.86±0.03)	223.76±9.22 (0.90±0.04)
Ovaries	28.11±1.69 (0.10±0.01)	27.93±0.83 (0.08±0.02)	28.60±1.38 (0.10±0.01)	29.80±2.98 (0.11±0.01)	19.24±2.19* (0.08±0.01)

Peruvioses A and B: Sucrose esters isolated from *Physalis peruviana* L. calyces. MMC: Mitomycin C. After mice sacrifice, organ weight was recorded (mg) and a relative organ-to body weight was calculated and expressed as percentage. Data is expressed as mean ± ESM, from two independent experiments. **P*<0.05, ***P*<0.01, *****P*<0.0001 ANOVA from control group.

In the case of MMC-treated mice (0.5 mg/Kg/day), the weight of liver, kidneys, heart, and lungs showed no significant differences with control group. However, the absolute weight of spleen and ovaries from MMC-treated female mice was significantly lower than control mice (Table 6.1, $P<0.05$). Similarly, absolute and relative testes weight from MMC-treated male mice were remarkably reduced ($P<0.0001$). With regard to female mice, histological analysis of spleen revealed that some animals presented a slight increase in the red pulp area without disruption of splenic architecture (Figure 6.2B). It was also found a minor loss of developing follicles in the ovaries, in absence of other atrophy associated lesions (Figure 6.2B). On the other hand, histological evaluation of testes revealed alteration in the structure of seminiferous epithelium with germ cell degeneration characterized by disorganized arrangement of germ cells in the tubules, presence of vacuoles and slight

necrosis (Figure 6.2A). Our results are similar to recently reported effects of MMC on testes of albino mice.[34]

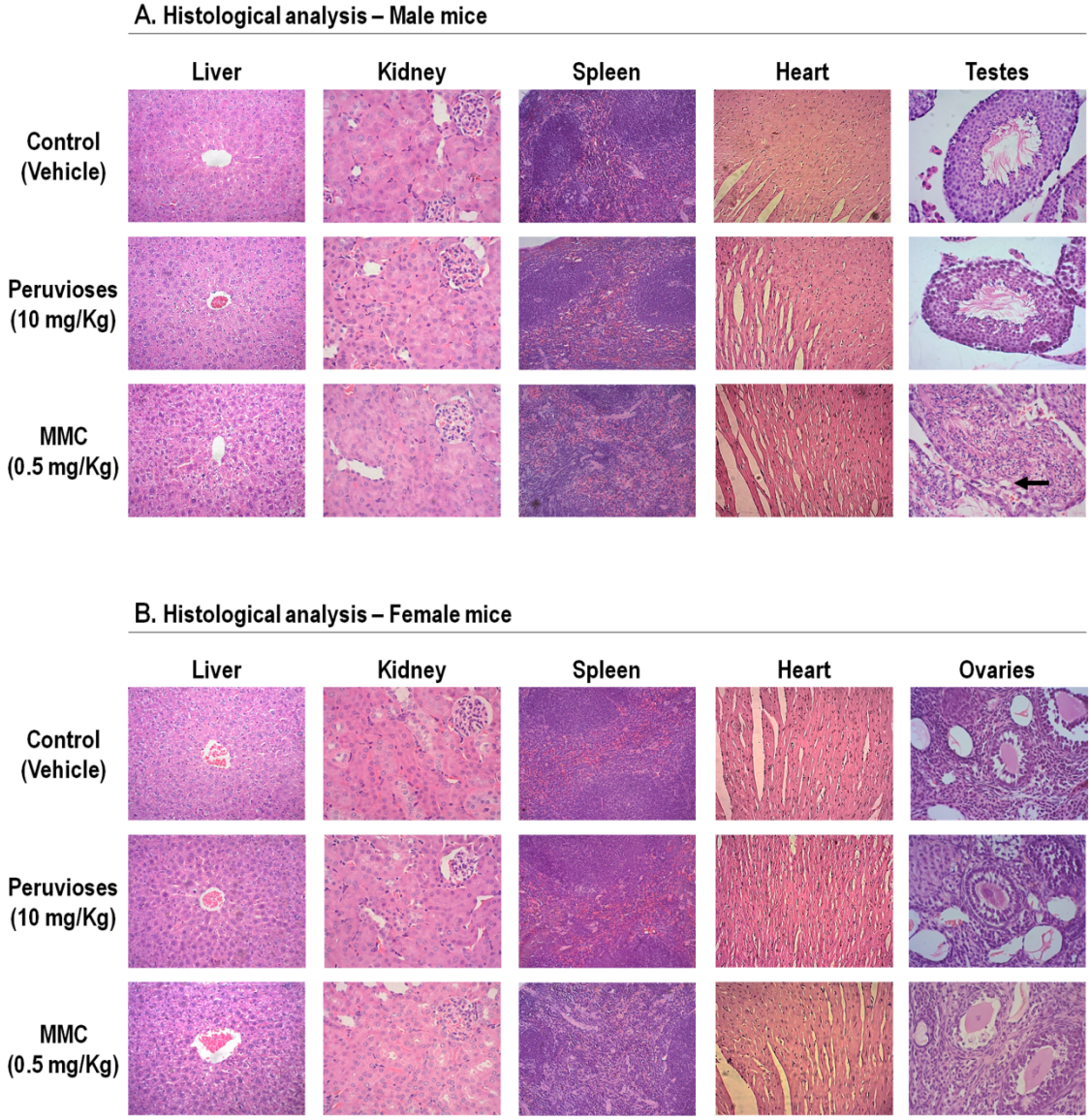


Figure 6.2. Sucrose esters from *Physalis peruviana* did not alter the histological architecture of liver, kidney, spleen, heart or gonads. CD-1 (ICR) mice received peruvioses A and B (10 mg/Kg/day, ip), mitomycin C (MMC, 0.5 mg/Kg/day, ip), or vehicle for 28 days. Organs (liver, kidneys, spleen, heart, and gonads) were harvested, stained with hematoxylin and eosin (H&E), and analyzed by a blinded researcher. Representative pictures of male (A) and female (B) mice are shown. Black arrow is pointing vacuolization in testes of mice treated with MMC. Images from liver, spleen and heart were taken with 20X magnification. Images from kidney, testes and ovaries were taken with 40X magnification.

6.4.3 Biochemical and urine parameters of mice were not affected by repeated administration of Sucrose esters from *Physalis peruviana*.

Following 28-day administration of the mixture of sucrose esters from *P. peruviana,* urine was collected and assessed with urinary test papers. Urinalysis did not reveal changes in parameters such as specific gravity, pH, protein, nitrite, urobilinogen, bilirubin, glucose, and the presence of occult blood or ketones (data not shown).

Table 6.2. Effect of sucrose esters from *Physalis peruviana* on biochemistry serum parameters in CD-1 (ICR) mice.

Treatment	Serum Biochemical Parameters			
	Control (Vehicle)	Peruvioses A and B (mg/Kg)		MMC (0.5 mg/Kg/day)
		5	10	
Males *n*=5-10				
Glucose (mg/dL)	67.20±5.82	61.40±4.91	63.40±3.07	58.20±5.33
Albumin (g/L)	1.86±0.22	1.50±0.11	1.64±0.14	1.96±0.21
Urea Nitrogen (mg/dL)	49.75±5.12	47.79±1.88	40.58±1.34	42.56±4.73
Creatinine (mg/dL)	0.48±0.06	0.47±0.04	0.47±0.03	0.39±0.01
ALPa (IU/L)	94.43±12.73	81.20±14.49	69.80±9.46	98.00±15.82
ASTb (IU/L)	121.17±16.90	98.38±9.50	98.38±9.51	142.50±7.05
ALTc (IU/L)	58.57±6.89	43.25±2.32*	44.80±2.73*	50.40±2.16
Females *n*=5-10				
Glucose (mg/dL)	60.00±4.04	52.40±5.48	50.75±5.98	53.20±4.48
Albumin (g/L)	1.99±0.13	1.87±0.16	1.85±0.19	2.03±0.18
Creatinine (mg/dL)	0.53±0.05	0.42±0.04	0.48±0.04	0.34±0.02*
ALP (IU/L)	105.00±9.74	81.80±13.15	77.50±11.85	98.20±8.94
AST (IU/L)	108.86±8.05	126.00±9.84	152.13±26.08	154.80±10.91
ALT (IU/L)	41.13±2.85	48.10±3.10	58.71±9.13	51.00±3.45

Peruvioses A and B: Sucrose esters isolated from *Physalis peruviana* L. calyces. MMC: Mitomycin C. Data is expressed as mean ± ESM, from two independent experiments. **P*<0.05, ***P*<0.01, *****P*<0.0001 ANOVA from control group. [a.] Alkaline Phosphatase. [b.] Aspartate aminotransferase. [c.] Alanine aminotransferase.

The serum biochemistry data are summarized in Table 6.2. Administration of the mixture of peruvioses A and B (5 and 10 mg/Kg/day, ip) did not caused significant changes in parameters such as glucose, albumin, urea nitrogen, and creatinine levels when compared to control group. The serum levels of liver function parameters such as ALT, AST and ALP, were found to be modestly modified when mice were treated with sucrose esters from *P. peruviana,* with values within normal laboratory ranges for CD-1 (ICR) mice.[35] However, significant reduction of ALT levels was observed in male mice at both dosage levels. The effect of MMC (0.5 mg/Kg/day, ip) was also investigated. Similarly, most of the serum biochemical parameters were not modified when compared to control mice. However, creatinine levels were significantly diminished in MMC-treated female mice (Table 6.2, $P< 0.05$). Although creatinine levels were within normal ranges, this result might be related to the reduction in body weight and consequently muscle mass.

6.4.4 Sucrose esters did not alter leukocyte subsets proportions or increase the number of MN-peripheral erythrocytes

To assess the distribution of leukocyte subsets, we performed a differential count on blood smears from sucrose esters treated mice, as well as control animals. No test article-related effects were observed in the percentage of lymphocytes, including reactive and immature cells, neutrophils, monocytes, eosinophil and basophiles (Table 6.3). In the case of MMC-treated mice, changes were only observed in eosinophil counts, with a reduced percentage in male mice when compared to control group (P<0.05).

Table 6.3. Effect of sucrose esters from *Physalis peruviana* on hematological parameters in CD-1 (ICR) mice.

Treatment	White Cell Count (%)				
	Control (Vehicle)	Peruvioses A and B (mg/Kg/day)			MMC (0.5 mg/Kg/day)
		2.5	5	10	
Males *n*=5-10					
Lymphocytes	62.93±2.73	68.59±5.34	65.83±1.45	66.85±1.82	59.22±2.63
Reactive Lymphocytes	4.00±0.64	1.98±0.15	4.00±0.88	3.44±0.63	5.22±1.55
Immature Lymphocytes	1.22±0.34	2.41±0.48	0.88±0.35	1.56±0.48	0.11±0.11
Monocytes	4.22±0.62	3.29±0.39	5.00±0.47	4.32±0.66	4.67±1.20
Neutrophils	23.38±2.47	19.11±3.85	19.22±0.91	19.48±1.31	29.22±2.36
Eosinophils	3.29±0.66	2.41±0.75	3.18±0.63	2.63±0.49	0.56±0.34*
Basophils	1.28±0.33	2.20±0.54	1.50±0.25	1.50±0.34	1.00±0.55
Females *n*=5-10					
Lymphocytes	73.02±2.04	68.20±2.39	72.40±1.99	72.48±2.60	76.21±1.19
Reactive Lymphocytes	1.44±0.38	2.09±0.37	1.21±0.32	1.95±0.27	1.46±0.34
Immature Lymphocytes	1.44±0.42	2.30±0.33	1.25±0.42	1.50±0.41	0.44±0.44
Monocytes	6.83±1.10	2.80±0.31	7.05±1.18	6.12±1.06	6.66±1.20
Neutrophils	14.10±1.59	19.30±2.06	14.83±1.57	14.68±1.92	13.11±0.61
Eosinophils	2.33±0.36	2.20±0.46	2.21±0.46	2.17±0.31	2.11±0.61
Basophils	0.78±0.22	2.00±0.28*	1.05±0.27	1.11±0.31	0.00±0.00

Peruvioses A and B: Sucrose esters isolated from *Physalis peruviana* L. calyces. MMC: Mitomycin C. Data is expressed as mean ± ESM, from two independent experiments. *P<0.05, ANOVA from control group.

In vivo rodent MN assay has been widely used to evaluate genotoxicity of chemicals.[36] The results of MN formation in peripheral erythrocytes are shown in Figure 6.3. As can be seen, treatment with peruvioses A and B did not induced significant changes in the number of both MN-PCE and MN-NCE, regardless the sex or dosage level. Conversely, male and female mice treated with MMC (0.5 mg/Kg/day) exhibited a remarkable induction of MN formation in PCE and NCE when compared to control group (Figure 6.3B), together with a moderate increase of PCE/NCE ratio, with significant differences only for female animals (P<0.01 from control group).

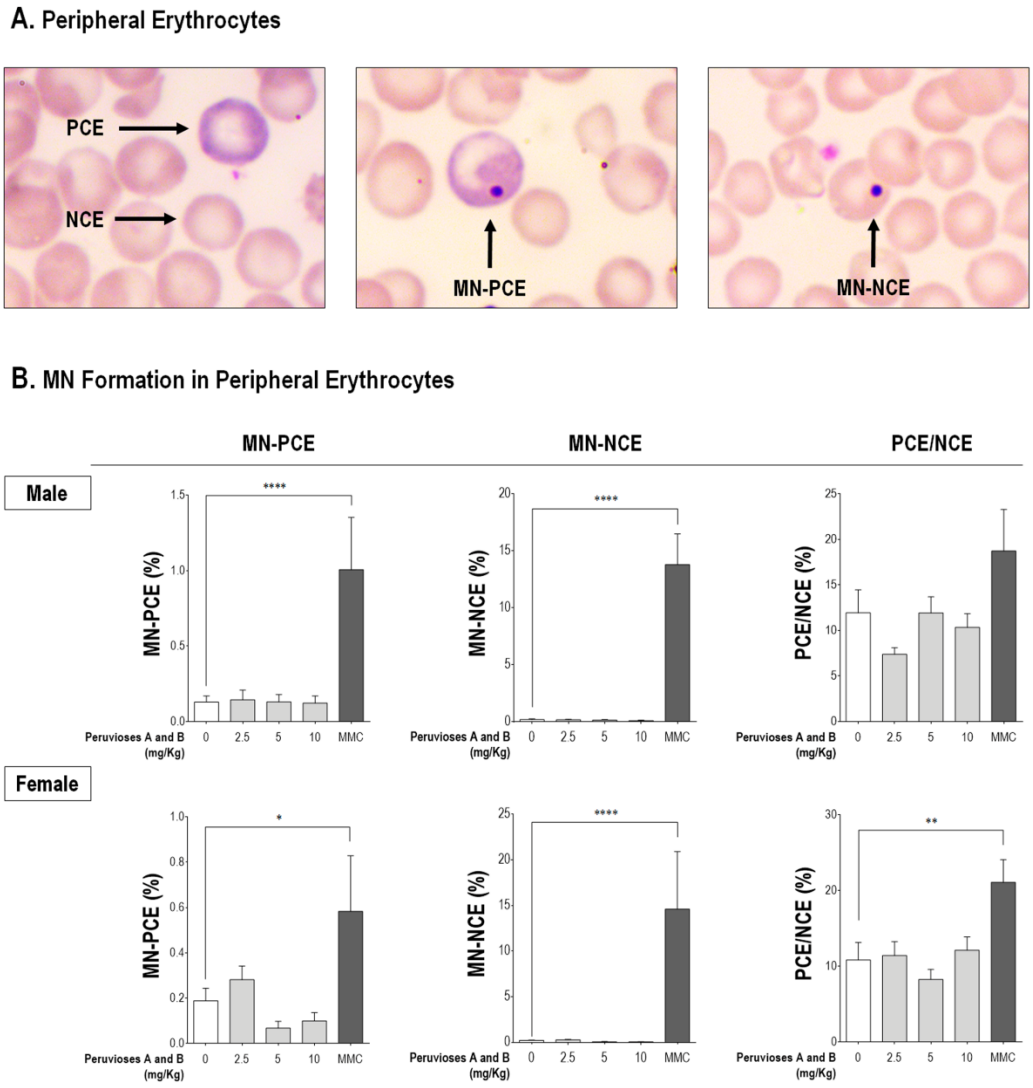


Figure 6.3. Treatment with sucrose esters from *P. peruviana* did not increase the frequency of micronucleated peripheral erythrocytes. CD-1 (ICR) mice received peruvioses A and B (2.5, 5, and 10 mg/Kg/day), mitomycin C (MMC, 0.5 mg/Kg/day, ip), or vehicle for 28 days. Smears from peripheral blood were prepared and erythrocyte population was assessed. (A) Representative pictures of polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE), micronucleated PCE (MN-PCE), and micronucleated NCE (MN-NCE); (Wright's Stain; 100X). (B) Frequency of MN-PCE and MC-NCE, as well as PCE/NCE ratio. Results are representative of two independent experiments, and are expressed as the mean ± S.E.M. (n=5-10 per group). (*) P<0.05, (**) P<0.01, and (****) P<0.0001 vs. control group.

6.4.5 Treatment with sucrose esters from *Physalis peruviana* did not increase the frequency of MN-PCE in bone marrow smears.

The results obtained from the bone marrow MN assay of mice treated with the mixture of peruvioses A and B for 28 days are presented in Figure 6.4. Sucrose esters did not induce significant changes in the number of MN-PCE or PCE/NCE ratio. As expected, animals treated with MMC showed a high frequency of MN-PCE in bone marrow cells when

compared to the control (P<0.0001). Moreover, the PCE/NCE ratio was moderately reduced in MMC-treated mice, with significant differences only for female animals (P<0.01 from control group), which indicates bone marrow toxicity.

6.4.6 Administration of sucrose esters from *Physalis peruviana* did not promoted genotoxic effects

To determine whether sucrose esters from *P. peruviana* induce DNA damage, we employed neutral comet assay to evaluate peripheral blood cells. As shown in Table 6.4, mice treated with vehicle presented a basal level of DNA damage that was not significantly modified by the treatment with the mixture of peruvioses A and B (10 mg/Kg, ip), regardless of the analysis method (manual vs. automatized).

On the other hand, Ames test revealed no significant changes in the number of revertant colonies when TA100 bacteria were treated with peruvioses mixture (30 μg/mL) in comparison with spontaneous revertant colonies from the control group, in the presence and absence of S9 metabolic activation system (data not shown).

6.4.7 Hepatic gene expression of steatosis markers was not modified by treatment with *Physalis peruviana.*

Given the fatty liver phenotype observed in response to administration of certain sucrose esters, as previously reported by [37]; six genes that are representative markers of steatosis (Cd36, Fasn, Lpl, Ppara, Scd1, and Srebf1) were evaluated to analyze the hepatic effects induced by sucrose esters from *P. peruviana*, when administered for a 28-day period. RT-PCR analysis revealed no significant changes in the hepatic expression of marker genes in mice treated with peruvioses when compared to control group (Figure 6.5).

Table 6.4. DNA damage measured by neutral comet assay in peripheral blood cells from CD-1 (ICR) mice treated with sucrose esters from *Physalis peruviana.*

Treatment	Frequency Comets 2,3 and 4	% Tail DNA	Tail Moment	Olive Tail Moment (OTM)
Control				
Male (n=5)	2.13±0.53	21.80±1.49	5.07±0.29	3.01±0.17
Female (n=5)	1.65±0.58	20.56±2.04	4.87±0.68	3.03±0.36
Total	1.89±0.39	21.18±1.24	4.97±0.36	3.02±0.19
Peruvioses A and B				
Male (n=5)	2.18±0.36	17.63±1.70	4.72±0.58	2.99±0.31
Female (n=5)	3.63±0.83	17.46±1.54	4.31±0.70	2.80±0.40
Total	2.90±0.46	17.54±1.11	4.51±0.45	2.89±0.25

Peruvioses A and B: Sucrose esters isolated from *Physalis peruviana* L. calyces, administered daily (10 mg/Kg/day, ip). Peripheral blood was mixed with low-melting point agarose and analyzed using neutral comet assay. Slides were scored visually by three blinded investigator to assign a comet score (0-4). Percentage of DNA on tail (%Tail DNA), Olive Tail Moment (OTM) and Tail Moment were calculated by analyzing at least 1000 comets per slide (2000 comets/animal) using OpenComet software [33]. Data are expressed as mean ± ESM.

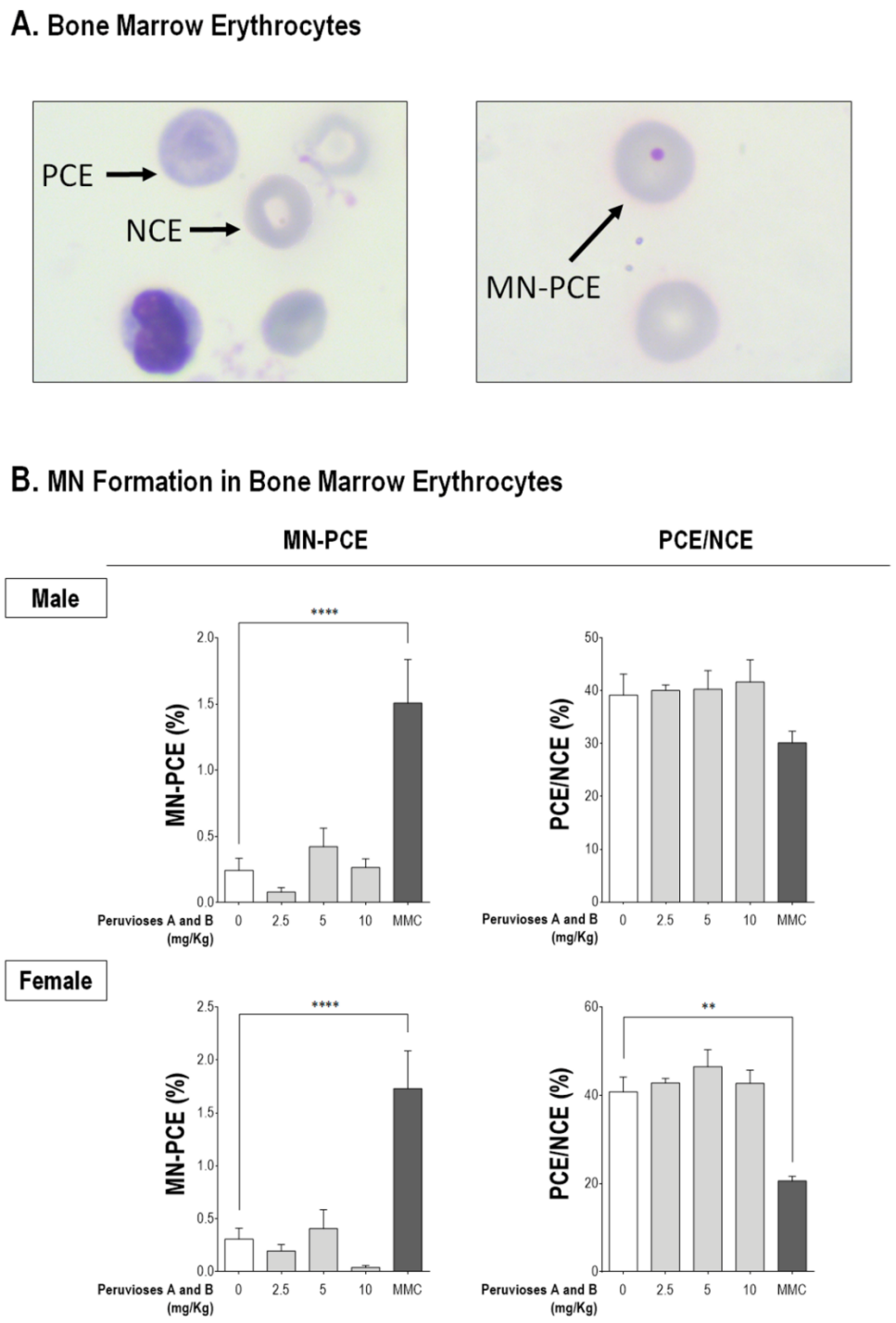


Figure 6.4. Sucrose esters from *Physalis peruviana* did not increase the frequency of micronucleated bone marrow erythrocytes. CD-1 (ICR) mice received peruvioses A and B (2.5, 5, and 10 mg/Kg/day), mitomycin C (MMC, 0.5 mg/Kg/day, ip), or vehicle for 28 days. Smears from bone marrow were prepared and erythrocyte population was assessed. (A) Representative pictures of polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE), and micronucleated PCE (MN-PCE); (Wright's Stain; 100X). (B) Frequency of MN-PCE and PCE/NCE ratio. Results are representative of two independent experiments, and are expressed as the mean ± S.E.M. (n=5-10 per group). (**) P<0.01, and (****) P<0.0001 vs. control group.

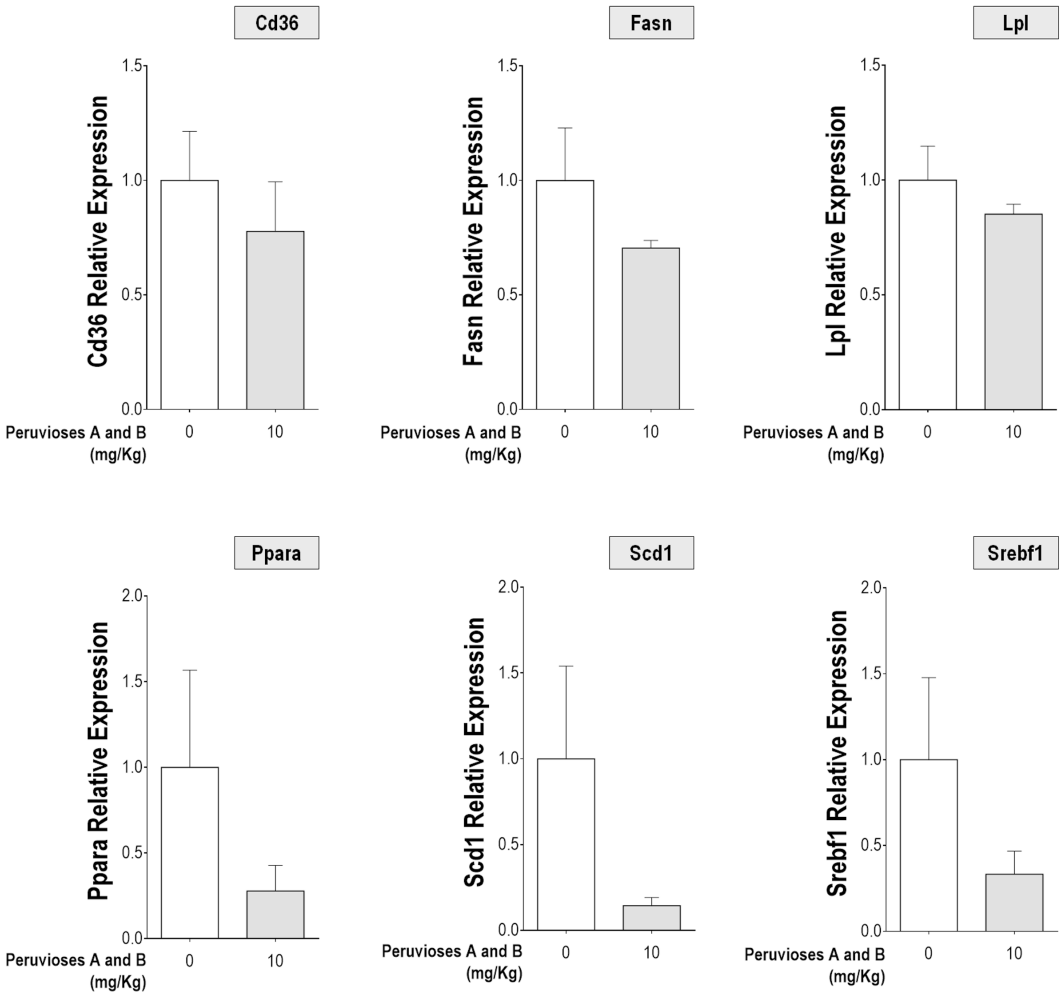


Figure 6.5. Gene expression of hepatic steatosis markers was not modified by administration of sucrose esters from *Physalis peruviana*. CD-1 (ICR) mice received peruvioses A and B (10 mg/Kg/day) or vehicle for 28 days. mRNA was extracted from liver of male mice, and six genes (Cd36, Fasn, Lpl, Ppara, Scd1, and Srebf1) were analyzed by RT-PCR. Results are expressed as the mean ± S.E.M. (n=5 per group).

6.5 Discussion

Sucrose esters are non-ionic emulsifiers that play important roles in food, cosmetics, and pharmaceutical industries.[15] Although considered safe and environmentally-friendly, information on the toxicity of these compounds is scarce, particularly when administration routes different to oral exposure are employed.[15, 38] Here, we show compelling evidence of the safety of peruviose A and B, sucrose esters isolated from *P. peruviana* calyces, in a 28-day repeated-dose study using intraperitoneal administration.

Aside from the variety offered by the industry, a vast collection of sucrose esters are present naturally in plants. Indeed, a unique feature of the glandular trichomes of plants

from the Solanaceae family, is that they produce glucose and sucrose esters, which have been shown to possess insecticidal, antifungal, and antibacterial properties.[23] *Physalis*, one of the best known genus of this family for its economic and ethnopharmacological importance, is also recognized as an abundant source of sucrose esters.[12] So far, twenty sucrose esters have been isolated from *P. viscosa*, *P. nicandroides* var attenuata, *P. sordida*, *P. solanaceus*, *P. peruviana*, *P. neomexicana*, and *P. philadelphica* [11, 13, 14, 39-41]. Within the pharmacological activities reported for these secondary carbohydrates, their anti-inflammatory and antibacterial effects appear to be the most important.[12] Noteworthy, these studies employed parenteral routes of administration, either topical or intraperitoneal, mainly to mimic the ethnopharmacological application or avoid gastrointestinal degradation of test compounds.[7, 11, 13] Therefore, to optimize the safe employment of *Physalis* species and their sucrose esters, toxicological studies are compulsory.

In this study, we evaluated the toxicological effect of the mixture of peruvioses A and B, from *P. peruviana* calyces, which were previously identified as promising anti-inflammatory agents.[11] We employed a sub-acute model of exposure where male and female CD-1 (ICR) mice were treated with peruvioses at doses of 2.5, 5, and 10 mg/Kg/day for 28 consecutive days. The highest dose (10 mg/Kg) corresponds to 2-4 times the effective dosage levels of sucrose esters in sub-acute and chronic models of chemically induced colitis (unpublished data). Administration of test compounds was performed by intraperitoneal route to guarantee complete access of the tested compounds to the general circulation. The noxious response (writhing and piloerection) that occasionally occurred after injection of test compounds (10 mg/Kg) was not unexpected, since we previously found this effect when treating mice at higher doses.[11] However, the occurrence, magnitude, and latency of discomfort after administration with peruvioses A and B (10 mg/Kg) was significantly less severe than the previously studied dosages (≥100 mg/Kg), where acute toxicological effects of sucrose esters resulted in pronounced pain, piloerection, and hypoactivity.[11] Our results indicate that this local, and transient noxious response of sucrose esters might be related to an irritant activity, since solutions were sterile and isotonic.[42] Nevertheless, no signs of corrosive reactions such as necrosis, bleeding, or discoloration; peritonitis; extravasation and liquid accumulation; or adhesions were found in the abdominal or thoracic cavities when performing the gross necropsy. Thus, intraperitoneal administration of peruvioses A and B (10 mg/Kg) is considered to be safe.

With regard to general toxic effects, mice treated with peruvioses A and B did not exhibited deterioration of their general condition, or differences in food consumption or body weight, during the 28-days treatment period, regardless of dosage level or sex. Macroscopic examination revealed no treatment related changes on vital organs weight or appearance.

Moreover, histological examination showed no alterations on any of the animals treated with peruvioses when compared to control group.

In our previous acute toxicity study, administration of peruvioses in doses from 215 to 300 mg/Kg produced toxic effects in a dose dependent-manner, thus they are classified in GHS category III (>50-300) according to OECD-423 guideline.[43] However, further analysis did not found abnormalities on the gross morphology and histology of liver and kidneys, key organs that metabolize and eliminate toxic substances.[11] These organs are especially important when studying sucrose esters administered by parenteral routes since previous intravenous dosing studies with rats and monkeys, demonstrated that if certain sucrose ester were absorbed intact, it would be rapidly taken up by the liver, systemically metabolized, and slowly eliminated through the bile and urine.[15, 44] In the present study, the values of functional biochemical parameters for liver (AST and ALP) and kidney (urea and creatinine) were not changed after sub-acute administration of peruvioses A and B, when compared to control group. Interestingly, ALT levels were significantly decreased in male mice treated with higher doses (5 and 10 mg/Kg). These levels were not considered to be pathological since they were within the normal biological ranges for CD-1 (ICR) mice.[35] Taken together, our evidence suggests that liver and kidney function are not affected by administration of sucrose esters from *P. peruviana*.

Remarkably, several independent studies have demonstrated the amelioration of chemically induced toxicity on liver, kidneys, and testicles by oral administration of extracts from leaves, roots, and fruits of *P. peruviana.* Such bioactivity is related to the reduction of histological damage; oxidative stress markers (malondialdehyde, superoxide dismutase, and nitric oxide); and serum biochemical markers (ALT, AST, ALP, bilirubin, creatinine, and urea).[45-49]

Assessment of the mutagenic and genotoxic effects of sucrose esters from *P. peruviana* is compulsory to support its safety. Consequently, after sub-acute administration of peruvioses A and B, the endpoints integrated were micronucleated erythrocytes in peripheral blood; micronucleated polychromatic erythrocytes in bone marrow; as well as DNA damage in peripheral blood cells, via Neutral Comet assay. In addition, to further investigate if peruvioses were capable of inducing mutations, Ames test was also employed.

The micronucleus (MN) test is one of the most widely applied assays to test new compounds, *in vivo* and *in vitro*, for regulatory purposes.[29, 50, 51] MN are formed during cell division, resulting either from chromosome breakage (clastogenic agents) or loss (aneugenic agents).[52] Data analysis revealed no increases in the MN-frequencies in peripheral or bone marrow erythrocytes of peruvioses-treated mice, regardless the dosage level. The negative controls were within our historical control database and the positive

control MMC produced a statistically significant increase in the frequency of MN-erythrocytes. Therefore, the validity of the test system was proven.

It is well-known that comet assay (single cell gel electrophoresis) is a simple, rapid, sensitive and quantitative method for measuring DNA damage, at the level of individual cells.[53, 54] Moreover, the combination of the comet assay and MN test is a useful method to detect genotoxic carcinogens that are undetectable with MN assay alone.[55] Thus, a comet assay was conducted to detect the *in vivo* genotoxic effect of sucrose esters mixture from *P. peruviana*. The results did not show significant increase in the analyzed parameters (frequency of comets, % tail DNA, or tail moment) due to repeated treatment with peruviose A and B. Hence, test compounds were judged to be negative in the comet assay.

For *in vitro* toxicology assessment of an herbal extract, Ames test is frequently the method of choice.[56] Mutagenicity measured by the bacteria reverse mutation test provides information about the tendency of a test substance to induce changes in DNA sequence.[57] Data analysis revealed strong mutagenicity activity of sodium azide and 2-anthramine, employed as positive control. In contrast, no evidence of mutagenic properties was found for peruvioses A and B. Although we did not employ the minimum number of bacterial strains recommended by the OECD [58], our results employing mammalian *in vivo* conditions are sufficient to provide unequivocal support of the lack of genotoxicity of sucrose esters from *P. peruviana*.

The accumulation of DNA lesions plays a pivotal role in carcinogenesis, an increase in the frequency of reversed bacteria [59], micronucleated cells [60], and comets [61] has been reported to predict accurately cancer risk. Therefore, our evidence supports the safety of chronic treatment with peruvioses A and B from *P. peruviana*, at the studied dosage levels. Nevertheless, additional *in vivo* studies employing longer periods of administration are recommended. Interestingly, studies of the chronic toxicity of sucrose esters, administered per oral, showed no adverse effects on the animals, and no evidence of increased frequency of tumors or other abnormalities.[15, 62, 63]

As mentioned before, all available data indicate that sucrose esters are harmless with current regulations supporting the safety of sucrose esters trough oral consumption, up to 40 g per Kg as acceptable daily intake.[15] However, the exposure route is expected to make a difference in bioavailability and metabolism of compounds and consequently in their toxicological effects. In fact, *in vivo* assays with intraperitoneal administration are thought to be more sensitive than those with per oral administration.[64] In the case of Peruvioses A and B, although administered intraperitoneally, our findings are similar to those seen after oral administration of other sucrose esters. From our point of view, this might be related to the fact that the first-pass through liver is shared by both routes of administration.[42] Indeed, previous reports demonstrated that in both humans and rodents,

administered sucrose esters are partially hydrolyzed in the gastrointestinal tract, presumably to sucrose, fatty acids, and partially esterified fatty acids, which are absorbed and metabolized largely in the liver [65].

One of the main drawbacks of this study is that the dosage range was narrower and dosage levels were lower, in comparison to those previously employed with oral gavage studies. Yet, they were selected to guarantee lack of toxicity of test compounds at therapeutic doses. In addition, bioavailability is higher by intraperitoneal administration, especially in the case of sucrose esters that are metabolized in the gastrointestinal tract. Nevertheless, we recommend that further studies with peruvioses should be conducted using a wider range of doses levels to establish no observed adverse effect levels (NOAEL).

In conclusion, this pre-clinical study demonstrated the lack of toxicological effects in CD-1 (ICR) mice, treated with peruvioses A and B from *P. peruviana*, for a 28-days period. Thus, our findings provide some assurance of the safety of sucrose esters administered by parenteral routes.

6.6 References

1. Puente, L.A., et al., *Physalis peruviana Linnaeus, the multiple properties of a highly functional fruit: A review.* Food Research International, 2011. **44**(7): p. 1733-1740.
2. Fischer, G., P. Almanza-Merchán, and D. Miranda, *Importancia y cultivo de la uchuva (Physalis peruviana L.).* Revista Brasileira de Fruticultura, 2014. **36**(1): p. 1-15.
3. Simbaqueba, J., et al., *Development and characterization of microsatellite markers for the Cape gooseberry Physalis peruviana.* PLoS One, 2011. **6**(10): p. e26719.
4. Fischer, G. and D. Miranda, *Uchuva (Physalis peruviana L.)*, in *Manual para el cultivo de frutales en el trópico*2012, Produmedios. p. 851-853.
5. Bravo, K., et al., *Influence of cultivar and ripening time on bioactive compounds and antioxidant properties in Cape gooseberry (Physalis peruviana L.).* J Sci Food Agric, 2015. **95**(7): p. 1562-9.
6. Fisher, G. and P. Lüdders, *Developmental changes of carbohydrates in Cape Gooseberry (Physalis peruviana L.) fruits in relation to the calyx and the leaves.* Agronomia Colombiana, 1997. **14**(2): p. 95-107.
7. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica, 2007. **27**(1): p. 110-5.
8. Castro, J., Y. Ocampo, and L. Franco, *Cape Gooseberry [Physalis peruviana L.] Calyces Ameliorate TNBS Acid-induced Colitis in Rats.* Journal of Crohn's and Colitis, 2015. **9**(11): p. 1004-1015.
9. Toro, R.M., et al., *Phytochemical analysis, antioxidant and anti-inflammatory activity of calyces from Physalis peruviana.* Nat Prod Commun, 2014. **9**(11): p. 1573-5.
10. Martinez, W., et al., *In vitro studies on the relationship between the anti-inflammatory activity of Physalis peruviana extracts and the phagocytic process.* Immunopharmacol Immunotoxicol, 2010. **32**(1): p. 63-73.
11. Franco, L.A., et al., *Sucrose esters from Physalis peruviana calyces with anti-inflammatory activity.* Planta Med, 2014. **80**(17): p. 1605-14.
12. Zhang, W.N. and W.Y. Tong, *Chemical Constituents and Biological Activities of Plants from the Genus Physalis.* Chem Biodivers, 2016. **13**(1): p. 48-65.

13. Perez-Castorena, A.L., M. Martinez, and E. Maldonado, *Labdanes and sucrose esters from Physalis sordida.* J Nat Prod, 2010. **73**(7): p. 1271-6.
14. Zhang, C.R., et al., *New antiinflammatory sucrose esters in the natural sticky coating of tomatillo (Physalis philadelphica), an important culinary fruit.* Food Chem, 2016. **196**: p. 726-32.
15. Zheng, Y., et al., *Sugar Fatty Acid Esters*, in *Polar Lipids* 2015, Elsevier. p. 215-243.
16. Zhao, L., et al., *In vitro antibacterial activities and mechanism of sugar fatty acid esters against five food-related bacteria.* Food Chem, 2015. **187**: p. 370-7.
17. Nava-Arzaluz, M.G., E. Piñón-Segundo, and A. Ganem-Rondero, *Sucrose Esters as Transdermal Permeation Enhancers*, in *Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement: Modification of the Stratum Corneum*, N. Dragicevic and I.H. Maibach, Editors. 2015, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 273-290.
18. Nelen, B.A.P., L. Bax, and J.M. Cooper, *Sucrose Esters*, in *Emulsifiers in Food Technology*2014, John Wiley & Sons, Ltd: Chichester, UK. p. 147-180.
19. Youan, B.B., A. Hussain, and N.T. Nguyen, *Evaluation of sucrose esters as alternative surfactants in microencapsulation of proteins by the solvent evaporation method.* AAPS PharmSci, 2003. **5**(2): p. E22.
20. Kurti, L., et al., *The effect of sucrose esters on a culture model of the nasal barrier.* Toxicol In Vitro, 2012. **26**(3): p. 445-54.
21. Ahsan, F., et al., *Sucrose cocoate, a component of cosmetic preparations, enhances nasal and ocular peptide absorption.* Int J Pharm, 2003. **251**(1-2): p. 195-203.
22. Kroumova, A.B.M., D. Zaitlin, and G.J. Wagner, *Natural variability in acyl moieties of sugar esters produced by certain tobacco and other Solanaceae species.* Phytochemistry, 2016. **130**: p. 218-227.
23. Eich, E., *Secondary Metabolites Derived from Fatty Acids and Carbohydrates*, in *Solanaceae and Convolvulaceae: Secondary Metabolites (A Handbook)*2008, Springer: Berlin, Heidelberg. p. 525-582.
24. OECD., *Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents*. OECD Guidelines for the Testing of Chemicals2008, Paris: OECD Publishing.
25. Ocampo, Y.C., et al., *Potential of Peruviose A and B from Physalis peruviana L calyces to treat Inflammatory Bowel Disease: In vivo and in vitro studies.* Planta Med, 2013. **79**(13): p. PB34.
26. Hamada, S., et al., *Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: Summary of the 13th Collaborative Study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)-Mammalian Mutagenicity Study Group (MMS).* Environ Mol Mutagen, 2001. **37**(2): p. 93-110.
27. Malone, M.H. and R.C. Robichaud, *A Hippocratic screen for pure or crude drug materials.* Lloydia, 1962. **25**(4): p. 320-332.
28. Kurien, B.T. and R.H. Scofield, *Mouse urine collection using clear plastic wrap.* Laboratory Animals, 1999. **33**(1): p. 83-86.
29. OECD, *Test No. 474: Mammalian Erythrocyte Micronucleus Test*2014, Paris: OECD Publishing. 21.
30. Schmid, W., *The micronucleus test.* Mutation Research/Environmental Mutagenesis and Related Subjects, 1975. **31**(1): p. 9-15.
31. Oshiro, Y., P.S. Balwierz, and G. Witz, *Micronucleus formation in mouse bone marrow cells in vivo in response to trans, trans-muconaldehyde.* Toxicology Letters, 2001. **121**(3): p. 159-166.

32. Cabarcas-Montalvo, M., J. Olivero-Verbel, and H. Corrales-Aldana, *Genotoxic effects in blood cells of Mus musculus and Iguana iguana living near coal mining areas in Colombia.* Science of The Total Environment, 2012. **416**: p. 208-214.
33. Gyori, B.M., et al., *OpenComet: An automated tool for comet assay image analysis.* Redox Biology, 2014. **2**: p. 457-465.
34. Ibrahim El -Alfy, N.Z., M.F. Mahmoud, and A.M. Abdullah, *Mitomycin-C Induced Genetoxic Effect in Lymphocytes and Histological Alterations in Testes of Male Albino Mice.* Egyptian Journal of Hospital Medicine, 2016. **62**: p. 18-27.
35. Quimby, F.W. and R.H. Luong, *Chapter 6 - Clinical Chemistry of the Laboratory Mouse*, in *The Mouse in Biomedical Research (Second Edition)*, J. Fox, et al., Editors. 2007, Academic Press: Burlington. p. 171-216.
36. Krishna, G. and M. Hayashi, *In vivo rodent micronucleus assay: protocol, conduct and data interpretation.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2000. **455**(1–2): p. 155-166.
37. IPCS. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anti-caking agents and certain other substances*. 1969; Available from: http://www.inchem.org/documents/jecfa/jecmono/v46aje51.htm.
38. Baker, I.J.A., et al., *Sugar fatty acid ester surfactants: Biodegradation pathways.* Journal of Surfactants and Detergents, 2000. **3**(1): p. 13-27.
39. Ovenden, S.P., et al., *Physaloside A, an acylated sucrose ester from Physalis viscosa.* J Nat Prod, 2005. **68**(2): p. 282-4.
40. Maldonado, E., et al., *Sucrose esters from the fruits of Physalis nicandroides var. attenuata.* J Nat Prod, 2006. **69**(10): p. 1511-3.
41. Cao, C.M., et al., *Withanolides and Sucrose Esters from Physalis neomexicana.* J Nat Prod, 2015. **78**(10): p. 2488-93.
42. Turner, P.V., et al., *Administration of substances to laboratory animals: routes of administration and factors to consider.* J Am Assoc Lab Anim Sci, 2011. **50**(5): p. 600-13.
43. OECD, *Test No. 423: Acute Oral toxicity - Acute Toxic Class Method*2002, Paris: OECD Publishing. 14.
44. Miller, K.W., et al., *Disposition of ingested olestra in the Fischer 344 rat.* Fundam Appl Toxicol, 1995. **24**(2): p. 229-37.
45. Arun, M. and V.V. Asha, *Preliminary studies on antihepatotoxic effect of Physalis peruviana Linn. (Solanaceae) against carbon tetrachloride induced acute liver injury in rats.* J Ethnopharmacol, 2007. **111**(1): p. 110-4.
46. El-Gengaihi, S.E., et al., *Chemical composition and biological evaluation of Physalis peruviana root as hepato-renal protective agent.* J Diet Suppl, 2013. **10**(1): p. 39-53.
47. Al-Olayan, E.M., et al., *The potential protective effect of Physalis peruviana L. against carbon tetrachloride-induced hepatotoxicity in rats is mediated by suppression of oxidative stress and downregulation of MMP-9 expression.* Oxid Med Cell Longev, 2014. **2014**: p. 381413.
48. Dkhil, M.A., et al., *The potential protective role of Physalis peruviana L. fruit in cadmium-induced hepatotoxicity and nephrotoxicity.* Food Chem Toxicol, 2014. **74**: p. 98-106.
49. Abdel Moneim, A.E., *Prevention of carbon tetrachloride (CCl4)-induced toxicity in testes of rats treated with Physalis peruviana L. fruit.* Toxicol Ind Health, 2016. **32**(6): p. 1064-73.
50. OECD, *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*2010, Paris: OECD Publishing. 23.
51. Cardinale, F., P. Bruzzi, and C. Bolognesi, *Role of micronucleus test in predicting breast cancer susceptibility: a systematic review and meta-analysis.* Br J Cancer, 2012. **106**(4): p. 780-90.

52. Fenech, M., *The in vitro micronucleus technique.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2000. **455**(1–2): p. 81-95.
53. Lin, H., N. Mei, and M.G. Manjanatha, *In Vitro Comet Assay for Testing Genotoxicity of Chemicals*, in *Optimization in Drug Discovery: In Vitro Methods*, W.G. Caldwell and Z. Yan, Editors. 2014, Humana Press: Totowa, NJ. p. 517-536.
54. Collins, A.R., *Measuring oxidative damage to DNA and its repair with the comet assay.* Biochim Biophys Acta, 2014. **1840**(2): p. 794-800.
55. Takasawa, H., et al., *Results of the International Validation of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens: Individual data for 1,2-dibromoethane, p-anisidine, and o-anthranilic acid in the 2nd step of the 4th phase Validation Study under the JaCVAM initiative.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2015. **786–788**: p. 144-150.
56. Sponchiado, G., et al., *Quantitative genotoxicity assays for analysis of medicinal plants: A systematic review.* J Ethnopharmacol, 2016. **178**: p. 289-96.
57. Ames, B.N., J. McCann, and E. Yamasaki, *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test.* Mutat Res, 1975. **31**(6): p. 347-64.
58. OECD, *Test No. 471: Bacterial Reverse Mutation Test*1997, Paris: OECD Publishing. 11.
59. Kirkland, D., et al., *Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or in vivo genotoxic activity? I. Reports of individual databases presented at an EURL ECVAM Workshop.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2014. **775–776**: p. 55-68.
60. Soeteman-Hernandez, L.G., G.E. Johnson, and W. Slob, *Estimating the carcinogenic potency of chemicals from the in vivo micronucleus test.* Mutagenesis, 2016. **31**(3): p. 347-58.
61. Brendler-Schwaab, S., et al., *The in vivo comet assay: use and status in genotoxicity testing.* Mutagenesis, 2005. **20**(4): p. 245-254.
62. Yoshida, M., et al., *Lack of toxicity or carcinogenicity of S-170, a sucrose fatty acid ester, in F344 rats.* Food Chem Toxicol, 2004. **42**(4): p. 667-76.
63. Takeda, K. and M. Flood, *Chronic toxicity and carcinogenicity of sucrose fatty acid esters in Fischer 344/DuCrj rats.* Regul Toxicol Pharmacol, 2002. **35**(2 Pt 1): p. 157-64.
64. Sekihashi, K., et al., *A comparison of intraperitoneal and oral gavage administration in comet assay in mouse eight organs.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2001. **493**(1–2): p. 39-54.
65. Noker, P.E., et al., *Metabolism of ^{14}C-labelled sucrose esters of stearic acid in rats.* Food Chem Toxicol, 1997. **35**(6): p. 589-95.

7 CHAPTER SEVEN. General Discussion

Naturally-occurring sucrose esters have been isolated mainly from the glandular trichomes of species belonging to the Solanaceae family.[1] Their taxonomic and ecological importance is well recognized, with studies showing that surface acylated sucrose metabolites are useful as chemotaxonomic markers to unveil confusing classification based on the morphology of the plant and molecular markers.[2, 3] Similarly, sucrose esters are considered an essential chemical mechanism for drought resistance [4] and defense against predators (pathogenic bacteria, fungi, and insects) through several mechanisms.[1, 5] From the pharmacological point of view, the isolation of these compounds in medicinal plants is a strong indicator of their potential bioactivity, which is remarkable in the case of *Physalis* genus, where the presence of sucrose esters in aerial parts, flowers, fruits, and calyces has been related to the anti-inflammatory activity described for their plant source.[6-8]

With regard to *P. peruviana*, the anti-inflammatory and immunomodulatory properties of extracts/fractions from the calyces have been well described.[9-11] Therefore, it is unsurprising that the main bioactive compounds from the ethereal extract of *P. peruviana* calyces were sucrose esters. Indeed, the extraction, fractionation and preliminary chemical screening (test for carbohydrates and esters) was conceived on the rationale that acylated sugar metabolites were abundant in aerial parts of this species. As a result, we obtained the first core finding of this doctoral thesis: the identification of two new sucrose esters named peruvioses A and B from the calyces of *P. peruviana* with anti-inflammatory activity.[12] Unfortunately, compounds were isolated as a mixture that could not be separated with the tools available to us and our collaborators. Reports of "inseparable" mixtures of acylated sucrose compounds are not unusual, since small differences in their structures and high abundance, together with their amphipathic nature, often leads to lack of resolution with standard separation techniques (preparative TLC, reverse HPLC).[13, 14] Although this is not the case for most recently published reports, it is obvious that this task was simplified by the separation of small amounts of compounds mixture, often with employment of time-consuming and low efficiency methods that are useful just for chemical characterization of secondary metabolites. Nevertheless, the technical experience gained with the development of this doctoral thesis has been useful to attempt new separation methods that are currently employed in new projects in our laboratory.

The isolation and structural elucidation of sucrose esters within *Physalis* genus, has flourished in the last decade, allowing the report of thirty compounds to date. A comprehensive revision of the occurrence and characterization of sucrose esters form *Physalis* genus, showed that a series of di- to penta-acylated compounds with low molecular aliphatic acids (up to C14) especially acetic, isobutyric (2-methyl-propionic; *iso*), 2-methylbutyric (*anteiso*), 3-methylbutyric (isovaleric; *iso*), 3-methyl-2-butenoic, decanoic

(capric), and dodecanoic (lauric) acids were determined to be responsible for *O*-acylation at (2, 3, and 4) glucose or (1' and 3') fructose moieties. Additionally, the presence of tigloyl group and tetradecanoic (myristic) acid was recently reported for compounds isolated from *P. alkekengi*.[15] Despite the wide distribution and importance of acyl sucrose in *Physalis*, the understanding of how acyl chains are attached to sucrose is still poor. However, the resemblance between the substituents and the positions of acylation reveals that sucrose esters from this genus might share the same biosynthetic pathway. In the specific case of peruvioses A and B, the positions of *O*-acylation were 2, 3, 4, and 3'; with isobutyric, 3-methylbutyric, and decanoic acid as substituents. Since both straight-chain and branched-chain fatty acids are present in the molecules, it is assumed that fatty acid synthase (FAS)-mediated and/or the α-keto acid elongation pathways could be involved in their synthesis, as previously reported for *Nicotiana* and *Solanum* species.[16, 17] Even though this doctoral thesis did not focus on the synthesis or the mechanisms regulating the biosynthetic pathways of sucrose esters from *P. peruviana*, such research is undoubtedly a pre-requisite to increase their potential for further exploitation and to guarantee their inclusion in the food/pharmaceutical industry.

On the other hand, the anti-inflammatory effect of the isolated mixture of peruvioses A and B was demonstrated using a rodent model of general inflammation - λ-carrageenan induced paw edema. This effect was related to a strong down-regulation of NO and PGE2 production, as proved by the experiments with LPS-stimulated peritoneal macrophages.[12] The evaluation of NO (iNOS) and PGE2 (COX-2) were frequent in this work since they are important for the management of unresolved chronic inflammation, and have also been described as the main molecular targets associated with the anti-inflammatory activity of acyl sucrose metabolites. Indeed, sucrose esters isolated from different plant sources have shown to inhibit significantly NO and PGE2 production in activated cells.[18, 19] In this regard, [7] recently reported that five sucrose esters isolated from *P. philadelphica* inhibited COX-1 and COX-2 enzymatic activity. To this point, with the identification of new sucrose esters from *P. peruviana*, and their further characterization, this doctoral thesis contributed with important chemical and pharmacological information concerning to the bioactivity of sucrose esters from *Physalis* genus.

Taking into account that immune system alterations and inflammation are involved in the pathophysiology of many chronic diseases, including inflammatory bowel disease (IBD), we started the evaluation of the effect of peruvioses A and B from *P. peruviana* calyces, as well as their parental total extract, in the rat model of colitis induced by TNBS. Although our previous investigations demonstrated that the anti-inflammatory effect was remarkable increased in the sucrose esters-enriched fraction when compared to the total extract [10], we decided to include the extract in this study to enhance the possibility of finding the metabolites with major impact on intestinal inflammation. In this sense, the critical component of this thesis relays on the study of calyces of *P. peruviana* as a whole (extract

+ isolated compounds), which in turn encompasses a greater and more ambitious objective from our research group: the development of naturally-derived treatments for IBD and colorectal cancer.

First, with the evaluation of the total ethereal extract it was proved that treatment with *P. peruviana* is effective to attenuate the intestinal inflammation induced by TNBS in rats. It is important to emphasize that, in this doctoral thesis, the colonic damage was assessed 3-days (preventive set-up) and 2-weeks (therapy set-up) after TNBS instillation, which allowed us to determine the effect of test extract/compounds on non-specific acute inflammation, as well as established/chronic inflammation. Regardless of the experimental layout, the severity of intestinal inflammation was assessed using macroscopic, histologic, and molecular parameters. With reference to the evaluation of *P. peruviana* total extract, it was found that as a consequence of its administration, the histological damage induced by TNBS to the intestinal epithelium was significantly reduced, which promoted a recovery in the extension of macroscopic lesions observed in colonic biopsies. The colonic weight/length ratio was also decreased in rats treated with extract, revealing an amelioration of the tissue edema. MPO activity was slightly reduced in both experimental settings, which suggests a lower neutrophil infiltration in comparison with untreated colitic rats. In addition, when the deregulated immune response was evaluated, the results showed that extract solely down-regulated the colonic levels of IL-1β and TNF-α. This was accompanied by the modulation of LPS-activated RAW264.7 macrophages and their production of NO at non-toxic concentrations (Figure 7.1).

On the other hand, the chemical characterization of *P. peruviana* extract revealed the pronounced presence of flavonoids and steroids, together with esters and carbohydrates, which are distinctive of sucrose esters. Taking into account the abundance of flavonoids in the total extract, as well as their well-known antioxidant properties [20]; we decided to evaluate their scavenging effect employing DPPH and ABTS methods. Interestingly, the total extract showed low antioxidant properties *in vitro* (IC_{50} greater than 500 μg/mL), which suggests that the *in vivo* effect of test extract is related exclusively to its anti-inflammatory and immunomodulatory properties. This is noteworthy, since many reports of traditionally employed extracts put special emphasis on their antioxidant activity, usually overestimating it, as a mechanism to reduce the inflammation induced by the enhanced production of reactive oxygen and nitrogen species (RONS), which destructive effects on DNA, proteins and lipids contribute to the progression of IBD.[21, 22]

Steroids were another important group of compounds in the extract of *P. peruviana* calyces, suggesting the presence of withanolides, a group of oxygenated C28 ergostane-type steroids lactones, recognized as the most frequently occurring constituents of the genus *Physalis*. Up to know, one hundred sixty nine (169) withanolides have been reported from this genus.[6] In the matter of *P. peruviana*, previous phytochemical studies have led to the

isolation of more than thirty (30) withanolides from the whole plant, aerial parts, or calyces.[6, 23] As withanolides are considered the distinctive compounds from *Physalis*, their biological activities have been studied thoroughly. Nevertheless, just until recently, withanolides were recognized as potent immunomodulatory and anti-inflammatory compounds, with ability to regulate the activation LPS-stimulated macrophages trough suppression of NF-κB, STAT3, and HO-1.[23-26]

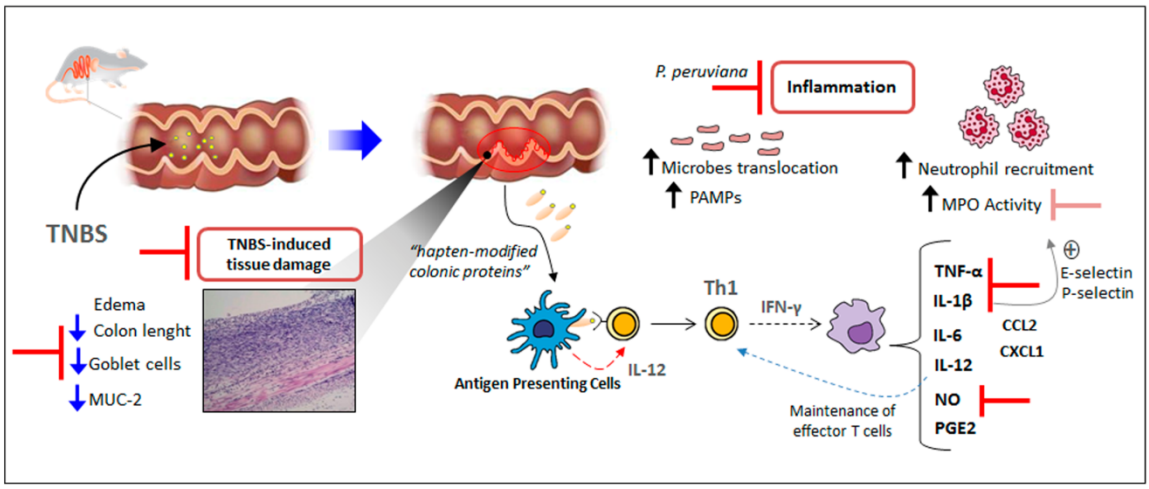


Figure 7.1. Molecular targets involved in the protective effect of the total extract of *Physalis peruviana* L. in TNBS-induced colitis. The inflammation induced by TNBS is mainly promoted by haptenization of host proteins, which provokes severe ulceration of the mucosa, colon shortening, and edema. At histological level, TNBS promotes extensive mucosa ulceration with goblet cells depletion and transmural infiltration on mononuclear cells and neutrophils recruitment which can be monitored by measurement of myeloperoxidase (MPO) activity. The deregulated immune response is induced by microbe's translocation to lamina propria, as well as by hapten-modified colonic proteins that are recognized by antigen presenting cells that in turns promote a Th1 response. This leads to IL-12 secretion and the induction of IFN-γ by activated T cells. IFN-γ stimulates macrophages to produce cytokines (IL-1β, IL-6, IL-12, and TNF-α), chemokines (CCL2 and CXCL1), and others mediators of inflammation (NO and PGE2). IFN-γ, TNF-α, and IL-12 act in synergy to maintain a positive feedback for Th1 response.[27] The total extract obtained from the calyces of *P. peruviana* L. significantly ameliorated the intestinal inflammation induced by TNBS in rats. This activity might be related with the reduction of the neutrophils migration and restoration of intestinal mucus layer, as well as the decrease of TNF-α, IL-1β and NO levels, which are considered crucial targets in IBD. Blunt-head red arrows show inhibition or down-regulation as a consequence of the treatment with *P. peruviana*. Abbreviations; CCL2: chemokine ligand 2, also referred to as monocyte chemoattractant protein 1 (MCP-1); CXCL1: chemokine (C-X-C motif) ligand 1; MPO: myeloperoxidase, MUC2: mucin 2, NO, nitric oxide; PAMPs: pathogen-associated molecular patterns; PGE2, prostaglandin E2; Th1: T helper 1.

Undoubtedly, the presence of withanolides in the total ethereal extract of *P. peruviana* calyces might be related with its biological activity at intestinal level, especially on the account of their potent immunomodulatory effect. However, their abundance in the test extract is drastically surpassed by sugar esters, specifically the mixture of peruvioses A and

B, isolated in a bioguided fractioning. Therefore, from our point of view withanolides are not the major anti-inflammatory compounds of *P. peruviana* calyces. Instead, the presence of withanolides might explain the elevated acute toxicity observed for the extract - LD_{50} 345.49 (311.03-379.95) mg/Kg, since this type of compounds produce severe effects as piloerection, diarrhea, and severe weight loss, at low dosage levels (LD_{50} below 100 mg/Kg in most cases).[28] In fact, it was noticed that rats treated with test extract did not recover from the weight loss induced by TNBS, even though it was ameliorating the intestinal inflammation.

So far, our data indicates that the beneficial effect of *P. peruviana* extract in TNBS colitis is related to a restoration of the intestinal mucosa as well as modulation of the immune response with reduction of TNF-α, IL-1β and NO levels, important mediators involved in the pathological response and symptoms of IBD. Therefore, it was established, for the first time, the potential of calyces of *P. peruviana* as a promising source of new natural-derived compounds to treat intestinal inflammation. This was a milestone on this doctoral thesis project, since its achievement indicated the advancement to a new phase involving the evaluation of the major bioactive compounds of the extract, peruvioses A and B.

As previously mentioned, the experimental layout designed to work with the total extract was also employed to assess the effect of the mixture of peruvioses. In the case of the preventive model, the administration of peruvioses produced a significant but modest beneficial effect, as evidenced by the reduction of macroscopic and histological damage. Similarly, the treatment with these sucrose esters produced a remarkable amelioration of established/chronic TNBS-induced inflammation, by reducing mortality rates and weight loss, along with a marked diminution of edema and extension/severity of macroscopic and histologic damage. Since the greatest therapeutic effect was noticed in the therapeutic set-up (two weeks treatment), it is assumed that the efficacy of peruvioses improves with longer periods of administration.

To investigate the mechanisms associated with the beneficial effect of sucrose esters from *P. peruviana*, experiments were focused in molecular markers of epithelial integrity and immunological response. Our results suggest that peruvioses significantly recovered the integrity of the colon in colitic rats, as indicated by the increased expression of MUC-2 (the primary constituent of the mucus layer in the colon), MUC-3 (a membrane-bound mucin), and TFF-3 (a bioactive peptide related with adaptation during injury, mucosal defense, and epithelial repair).[29-31] Accordingly, histological analysis with PAS staining, revealed that test compounds produced a significant increment in goblet cells, the main cellular source of MUC2 and TFF3. Goblet cells and their main secretory product, mucus, have long been poorly appreciated; yet recent discoveries have placed these cells at the center of mucosal homeostasis maintenance and representing a major cellular component of the innate defense system.[32-34]

A compromised mucosal barrier induces the translocation of microbial and food antigens' leading to immune activation, but it is the lack of resolution with anti-inflammatory mechanisms which starts and maintains the development of chronic intestinal inflammation. Therefore, molecular markers associated with altered immune response in IBD were assessed. It was found, that peruviose A and B from *P. peruviana* induced a strong immunoregulation that ameliorated the inflammation in TNBS-treated rats either acutely (preventive set-up) or in an established disease (therapeutic set-up). The preventive administration of sucrose esters significantly reduced the expression of iNOS, COX-2, TNF-α, and IL-10. Likewise, after two weeks of treatment, peruvioses were able to diminish the elevated expression of iNOS, COX-2, TNF-α, and IL-10; along with IL-1β, IL-6, and IL-17. Remarkably, all the genes modulated by peruvioses mixture are targets of the transcription factor NF-κB (Figure 7.2). In accordance, western blot experiments demonstrated the suppression of NF-κB activation in the intestinal tissue of colitic rats treated with test compounds. Thus, we propose that the immunomodulatory effects of sucrose esters might result from inactivation of NF-κB signal pathway.

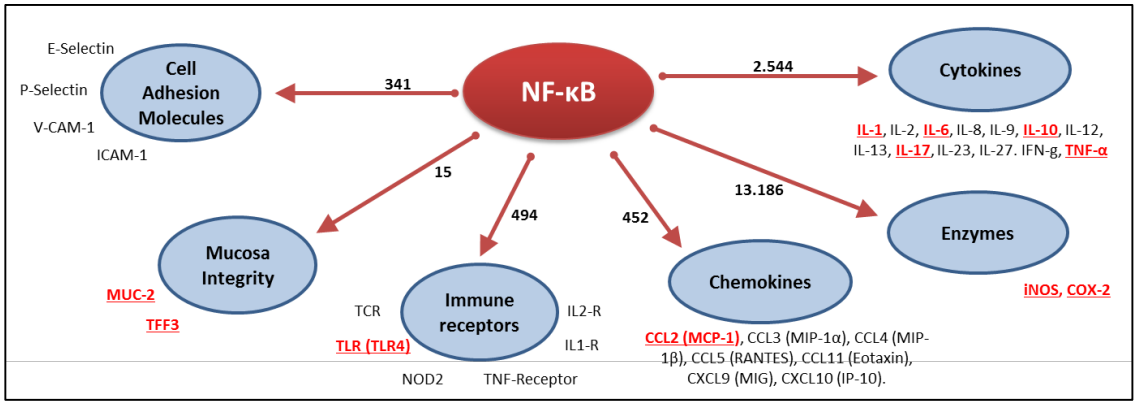


Figure 7.2. Regulation of transcriptional response by NF-κB. Nuclear factor-κB (NF-κB) is a central regulator of the transcriptional responses to a wide variety of physiological and environmental stimuli. Signals ranging from pro-inflammatory cytokines to stresses including reactive oxygen species (ROS), ultraviolet light and DNA double-strand breaks, to engagement of antigen receptors lead to activation of NF-κB. NF-κB transcriptional programs induce a wide variety of genes including survival factors, growth factors, cytokines, chemokines, numerous core mediators of adaptive and innate immunity, as well as micro-RNAs.[35] In this figure a limited number of targets related to inflammatory response are shown. The genes whose expression was modulated by peruvioses mixture are highlighted in red. Text-mining data is based on the Facta+ search of the keyword "NF-κB". Results are indicated by the number of hits found for each term; updated March, 2017.

Interestingly, iNOS, COX-2, TNF-α, and IL-10 appear as early molecular targets for peruvioses bioactivity, since their down-regulation leads the intestinal anti-inflammatory effect exerted by test compounds. It is important to remark that macrophages, key players in the induction/maintenance of intestinal inflammation, are the main cellular source of

these “early modulated” targets. In fact, the proportion of lamina propria macrophages is increased in IBD patients, as well as their responsiveness to TLR4 and NF-κB expression.[36-39] Additionally, activated macrophages promote the maintenance of Th17 cells (IFN+/IFN+IL17+, important source of IL-1β, IL-6 and IL-17).[40, 41] Thus, we further evaluated the effect of sucrose esters from *P. peruviana* on LPS-induced RAW 264.7 cells, which allowed us to confirm the strong modulation of macrophage activation with reduction of NO, PGE2, IL-6, TNF-α, and MCP-1 (Figure 7.3). This is consistent with the down-regulation of the macrophage's microbicide ability associated with reduction of pro-inflammatory cytokines levels (IL-6, TNF-α, and MCP-1) reported by [11], in murine macrophages infected with *L. panamensis* and treated with a hydroalcoholic fraction from *P. peruviana* calyces. Despite this evidence suggests a direct effect of peruvioses on intestinal macrophages activation, it is necessary to determine whether the reduction of pro-inflammatory mediators is a consequence of changes in phenotype and function of other cell populations that polarize macrophages activation *in vivo*.

With regard to the molecular mechanism involved in the anti-inflammatory activity of sucrose esters, little yet valuable information is available. First, Okabe *et al* reported that synthetic disaccharide esters were able to reduce TNF-α expression in an activator protein (AP)-1 dependent manner, without altering the nuclear translocation of NF-κB.[42] In contrast, Li *et al* reported the inhibitory activity of NF-κB by sucrose esters isolated from *Astragalus membranaceus*, which was correlated with a significant reduction of iNOS and ICAM-1 mRNA expression.[43] In agreement with the latter report, our experimental results indicates a strong modulation of pro-inflammatory enzymes (iNOS and COX-2), cytokines (IL-1, IL-6, IL-10, IL-17, and TNF-α), and chemokines (MCP-1) due to inhibition of NF-κB activation. Taken together, this doctoral thesis demonstrated that sucrose esters, particularly peruvioses from *P. peruviana*, exert their bioactivity by mechanisms which are beyond a simple enzymatic inhibition of COX-2 (PGE2) and iNOS (NO), as suggested by some authors.

While all the experimental evidence obtained from this doctoral thesis provides compelling evidence of the potential of sucrose esters from *P. peruviana* to treat IBD, it is obvious that more information is required to guarantee their employment in clinical trials. Since the evaluation of possible toxicological effects is mandatory, we evaluated the acute (24 hours) and sub-chronic (28-day repeated-dose) toxicity. Besides the high abundance of sucrose esters in the total extract of *P. peruviana* calyces, the results from acute toxicity demonstrated that peruvioses A and B were not the components responsible for the toxicity of total extract since their LD_{50} were remarkably similar (345.49 mg/Kg –Total extract Vs. 223.59 mg/Kg – Peruvioses). In fact, we found that toxicity signs were always milder when employing sucrose esters.

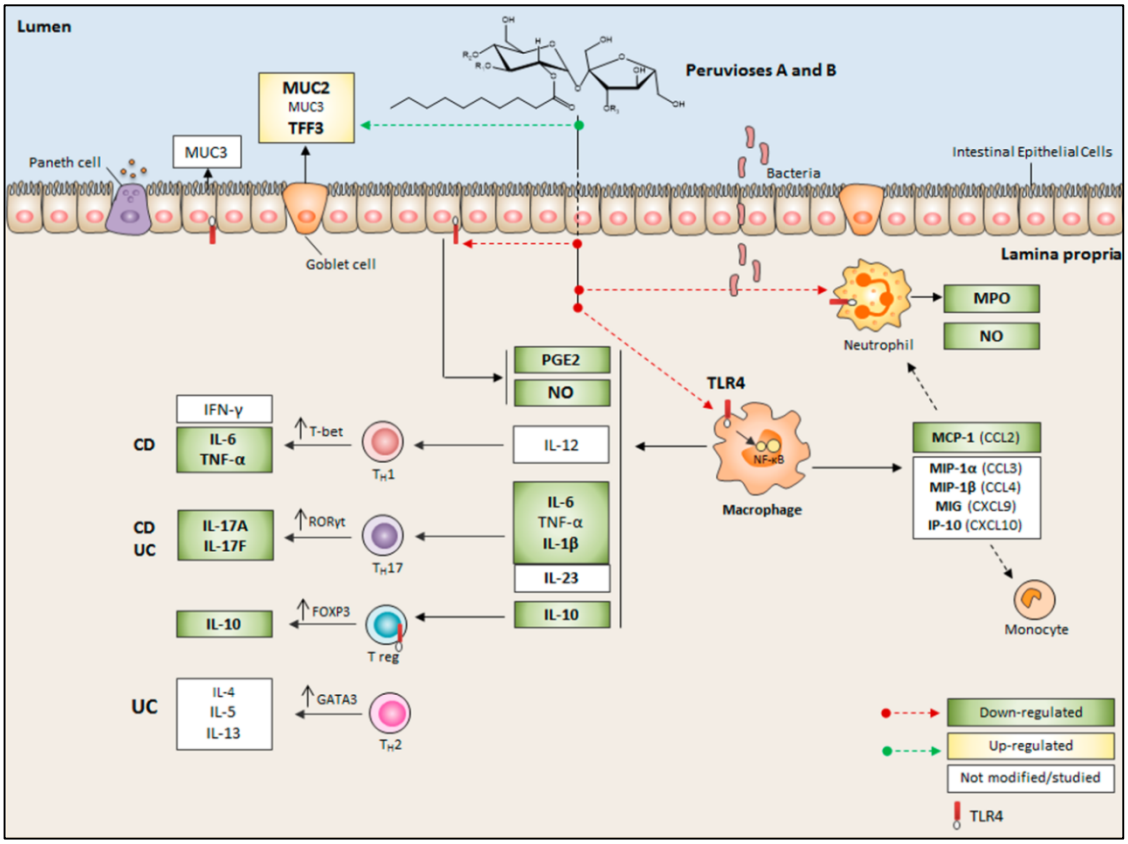


Figure 7.3 Model illustrating the molecular targets involved in the protective effect of sucrose esters from *Physalis peruviana* L. The mixture of peruvioses A and B attenuates the inflammation induced by TNBS by promoting the recovery of intestinal epithelium, with a significant increase in the number and function of goblet cells, together with a strong suppression of the activated immune response, reducing the expression of cytokines such as TNF-a, IL-1β, IL-6, and IL17, pro-inflammatory mediators such as NO and PGE2, and chemokines such as MCP-1; which results in ameliorating monocyte and neutrophil infiltration (reduced MPO activity). The anti-inflammatory effect observed is independent of IL-10 and probably is related to the inhibition of translocation of NF-κB. Macrophages are highlighted as the main cellular population modulated by peruvioses mixture. Further studies are required to clarify whether sucrose esters reduce pro-inflammatory mediators as a direct effect on macrophages activation or as a consequence of changes in other cell populations at intestinal level. Abbreviations: CD: Crohn's disease; IP-10: IFN-γ-inducible protein 10; MCP-1: monocyte chemoattractant protein 1; MIG: monokine induced by interferon gamma; MIP-1: macrophage inflammatory proteins α and β; MPO: myeloperoxidase; NF-κB: nuclear factor kappa B; NO, nitric oxide; PGE2, prostaglandin E2; mucosal effector T cells (T helper 1 (Th1), Th2 and Th17); TLR4: Toll like receptor-4; UC: ulcerative colitis. Red dotted arrows (green box) show inhibition or down-regulation while the green dotted arrow (yellow box) shows up-regulation. Proteins not affected by peruvioses or not studied are depicted in a white box.

The sub-chronic toxicity was studied following the Guidelines for the Testing of Chemicals from the Organization for Economic Cooperation and Development (OECD) and including clinical signs; food consumption; body and organ weights; hematological and biochemical parameters; macroscopic and microscopic examination of tissues; as toxicological end-points (Figure 7.4). In this case, peruvioses were remarkably well tolerated in therapeutic doses by test animals, with sporadic noxious response after ip administration and slightly decreased alanine transaminase (ALT) levels as the only major side effects. Otherwise, no differences were found between control and sucrose esters treated mice at macroscopic, histological, biochemical or hematological level. Moreover, a combination of micronucleus test and comet assay in peripheral blood cells, as well as Ames test showed that peruvioses did not induce genotoxic effects. As a whole, these results confirm the safety of peruvioses administration by parenteral route, at doses useful to treat inflammation.

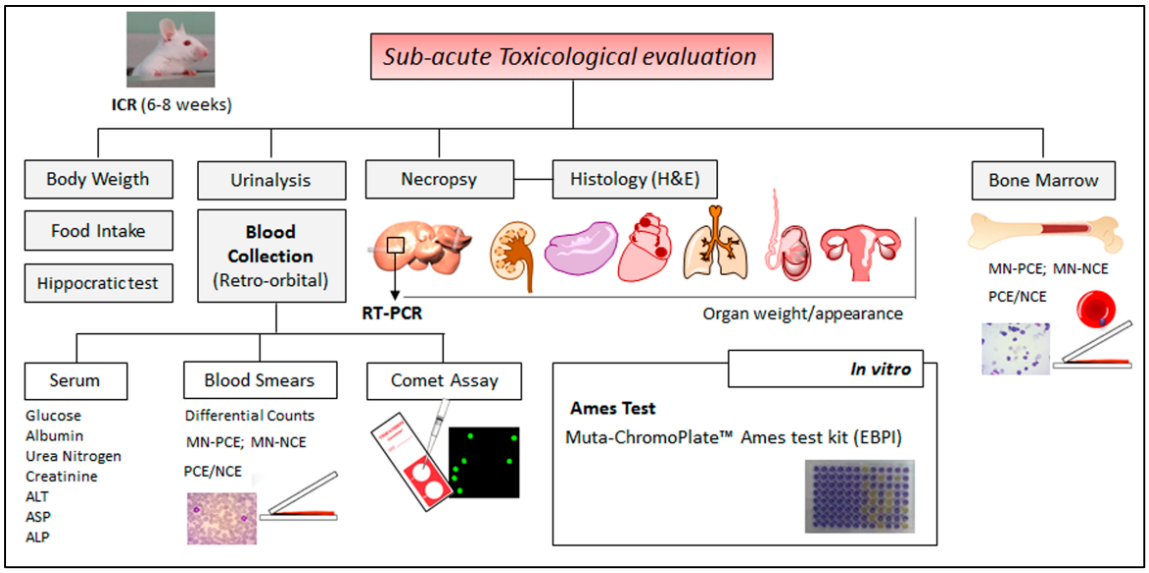


Figure 7.4 Schematic overview of the toxicological endpoints assessed to demonstrate the safety of sucrose esters from *P. peruviana*. CD1 (ICR) mice (6-8 weeks) were treated with the mixture of peruvioses A and B using therapeutic doses for a 28-days period. The safety of their administration was demonstrated employing macroscopic (body/organs weight, food intake, and Hippocratic test), histological, hematological, biochemical (serum and urine analysis), and molecular parameters. In addition, no genotoxic effects were induced by peruvioses either *in vivo* (micronucleus and comet assay) or *in vitro* (Ames test).

7.1 Concluding Remarks

In brief, the development of this doctoral thesis provided compelling pre-clinical evidence of the therapeutic potential of *Physalis peruviana* L. calyces to treat inflammation, specifically at intestinal level. These promising results encourage the development of a suitable pharmaceutical formulation that allows the clinical study of *P. peruviana* efficacy in IBD patients. In order to support this conclusion, the core findings of this thesis are summarized as follows:

- Two new sucrose esters named Peruvioses A and B were identified from the calyces of *P. peruviana.* These compounds have a significant effect in a rodent model of inflammation - λ-carrageenan induced paw edema, mainly through down-regulation of NO and PGE2 production.

- Total ethereal extract obtained from the calyces of *P. peruviana* significantly reduces the intestinal inflammation induced by TNBS in rats. This activity might be related with the reduction of the neutrophils migration into the colonic tissue and restoration of intestinal mucus layer, as well as the decrease of TNF-α, IL-1β and NO levels, which are considered crucial targets in IBD.

- The mixtures of peruvioses A and B, major bioactive compounds of the total extract of *P. peruviana*, ameliorates the symptoms and progression of colitis in the TNBS-induced model in rats, improving the epithelial recovery and modulating the cytokine unbalance associated with colitis, probably through suppression of NF-κB activation.

- Toxicological evaluation of peruvioses A and B from *P. peruviana*, revealed a lack of deleterious effects in mice, in a 28-days period. This study provided evidence of the safety of test compounds when administered by parenteral routes.

7.2 Challenges and Future Perspectives

Although all the experimental evidence obtained from this doctoral thesis provides compelling evidence of the potential of sucrose esters from *P. peruviana* to treat IBD, more experimental information is required to move from bench to bedside, which is particularly challenging when the molecule is obtained from a natural source. Apart from the standardization of the isolation and chemical characterization of parental extract/fractions (*i.e.*, HPLC or ^{1}H-NMR fingerprints) to guarantee consistency in bioactivity as well as a proper availability of peruvioses to engage in a clinical trial, it is essential to understand the mechanisms of sucrose esters biosynthesis in *P. peruviana*, to increase their potential for further exploitation and commercialization.

Strictly from the pharmacological point of view, it is mandatory to overcome the limitations related to the evidence presented in this thesis. First, we only demonstrated the efficacy of peruvioses A and B to treat colonic inflammation in one animal model. Thus, the bioactivity of test compounds should be determined in at least another model, preferably non-chemical induced. In this context, research is restricted in Colombia since such mice strains are not available nor can be imported at a reasonable price. Fortunately, a collaborative effort with Dr. Gerhard Rogler (University Hospital Zurich) and Dr. Xuhang Li (Johns Hopkins University) have allowed us the employment of C57BL6 mice to assess the effect of peruvioses A and B in preventing, treating, and ameliorating the progression of colitis in interleukin 10-negative ($IL10^{-/-}$) mice as well as mice treated with dextran sodium sulphate (DSS). Moreover, experiments using human tissue from IBD patients are necessary to provide some assurance of the effectiveness of peruvioses in clinic.

In addition, the pre-clinical evaluation of peruvioses will not be completed without the establishment of their pharmacokinetic profile, namely their bioavailability, metabolism, and elimination in both rodents and humans. Indeed, extremely well-designed pharmacokinetic studies are mandatory to determine suitable dosage levels. Complementarily, drug delivery of peruvioses also remains an important task to address since oral administration is preferred to treat IBD but intraperitoneal injection was used in all the experiments performed in this doctoral thesis. In this regard, systems such as nanoparticle or hydrogel formulations as carriers for oral colonic targeted drug delivery for peruvioses are interesting as valuable strategies to consider for future studies.

Another important approach that our research group intends to explore is the effectiveness of sucrose esters to prevent/treat IBD when administered from dietary sources. Sucrose esters might be present in the sticky coat from the surface of important food crops such as: naranjilla/lulo (*Solanum quitoense* Lam.); tamarillo (*Solanum betaceum* Cav.), sweet cucumber (*Solanum muricatum* Aiton); cocona/tupirum (*Solanum sessiliflorum* Dunal);

tomatoes (*Solanum lycopersicum* L.); eggplant (*Solanum melongena* L.); potato (*Solanum tuberosum* L.) in addition to gooseberry (*Physalis peruviana* L.) or wild gooseberry (*Physalis angulata* L.)*;* which are eaten as raw fruits or cooked vegetables. At the moment, we are working in the chemical characterization of *P. angulata*, as well on the assessment of the bioactivity of the extracts of some of these fruits. More aspects are planned to be explored since we consider food as an easily accessible, environmentally sustainable and economically viable source of immunomodulatory compounds.

In conclusion, it is clear from the results of this doctoral thesis that peruvioses A and B mixture is a promising preventive or therapeutic treatment for IBD. Nevertheless, considerable work remains to be done before the remarkable potential of these molecules is translated into a successfully clinical drug.

References

1. Eich, E., *Secondary Metabolites Derived from Fatty Acids and Carbohydrates*, in *Solanaceae and Convolvulaceae: Secondary Metabolites (A Handbook)*2008, Springer: Berlin, Heidelberg. p. 525-582.
2. King, R.R., R.P. Singh, and A. Boucher, *Variation in sucrose esters from the type B glandular trichomes of certain wild potato species.* American Potato Journal, 1987. **64**(10): p. 529-534.
3. Haliński, Ł.P. and P. Stepnowski, *Cuticular hydrocarbons and sucrose esters as chemotaxonomic markers of wild and cultivated tomato species (Solanum section Lycopersicon).* Phytochemistry, 2016. **132**: p. 57-67.
4. Fobes, J.F., J.B. Mudd, and M.P. Marsden, *Epicuticular Lipid Accumulation on the Leaves of Lycopersicon pennellii (Corr.) D'Arcy and Lycopersicon esculentum Mill.* Plant Physiol, 1985. **77**(3): p. 567-70.
5. Luu, V.T., et al., *O-acyl sugars protect a wild tobacco from both native fungal pathogens and a specialist herbivore.* Plant Physiol, 2017.
6. Zhang, W.N. and W.Y. Tong, *Chemical Constituents and Biological Activities of Plants from the Genus Physalis.* Chem Biodivers, 2016. **13**(1): p. 48-65.
7. Zhang, C.R., et al., *New antiinflammatory sucrose esters in the natural sticky coating of tomatillo (Physalis philadelphica), an important culinary fruit.* Food Chem, 2016. **196**: p. 726-32.
8. Perez-Castorena, A.L., M. Martinez, and E. Maldonado, *Labdanes and sucrose esters from Physalis sordida.* J Nat Prod, 2010. **73**(7): p. 1271-6.
9. Toro, R.M., et al., *Phytochemical analysis, antioxidant and anti-inflammatory activity of calyces from Physalis peruviana.* Nat Prod Commun, 2014. **9**(11): p. 1573-5.
10. Franco, L.A., et al., *Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces.* Biomedica, 2007. **27**(1): p. 110-5.
11. Martinez, W., et al., *In vitro studies on the relationship between the anti-inflammatory activity of Physalis peruviana extracts and the phagocytic process.* Immunopharmacol Immunotoxicol, 2010. **32**(1): p. 63-73.
12. Franco, L.A., et al., *Sucrose esters from Physalis peruviana calyces with anti-inflammatory activity.* Planta Med, 2014. **80**(17): p. 1605-14.
13. Neal, J.J., W.M. Tingey, and J.C. Steffens, *Sucrose esters of carboxylic acids in glandular trichomes of Solanum berthaultii deter settling and probing by green peach aphid.* J Chem Ecol, 1990. **16**(2): p. 487-97.

14. Buta, J.G., et al., *Sucrose esters from Nicotiana gossei active against the greenhouse whitefly Trialeuroides vaporariorum.* Phytochemistry, 1993. **32**(4): p. 859-864.
15. Zhang, C.Y., et al., *^{1}H NMR spectroscopy-guided isolation of new sucrose esters from Physalis alkekengi var. franchetii and their antibacterial activity.* Fitoterapia, 2016. **114**: p. 138-143.
16. Slocombe, S.P., et al., *Transcriptomic and reverse genetic analyses of branched-chain fatty acid and acyl sugar production in Solanum pennellii and Nicotiana benthamiana.* Plant Physiol, 2008. **148**(4): p. 1830-46.
17. Schilmiller, A.L., A.L. Charbonneau, and R.L. Last, *Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes.* Proc Natl Acad Sci U S A, 2012. **109**(40): p. 16377-82.
18. Wang, N., et al., *Bioactive sucrose esters from Bidens parviflora.* Phytochemistry, 2003. **62**(5): p. 741-6.
19. Li, W., et al., *NF-kappaB inhibitory activity of sucrose fatty acid esters and related constituents from Astragalus membranaceus.* J Agric Food Chem, 2013. **61**(29): p. 7081-8.
20. Kumar, S. and A.K. Pandey, *Chemistry and Biological Activities of Flavonoids: An Overview.* The Scientific World Journal, 2013. **2013**: p. 16.
21. Moura, F.A., et al., *Antioxidant therapy for treatment of inflammatory bowel disease: Does it work?* Redox Biology, 2015. **6**: p. 617-639.
22. Mosele, J., A. Macia, and M.J. Motilva, *Metabolic and Microbial Modulation of the Large Intestine Ecosystem by Non-Absorbed Diet Phenolic Compounds: A Review.* Molecules, 2015. **20**(9): p. 17429.
23. Sang-Ngern, M., et al., *Withanolides derived from Physalis peruviana (Poha) with potential anti-inflammatory activity.* Bioorg Med Chem Lett, 2016. **26**(12): p. 2755-9.
24. Sun, C.-P., et al., *Antiproliferative and Anti-inflammatory Withanolides from Physalis angulata.* J Nat Prod, 2016. **79**(6): p. 1586-1597.
25. Li, R.J., et al., *The Anti-inflammatory Activities of Two Major Withanolides from Physalis minima Via Acting on NF-kappaB, STAT3, and HO-1 in LPS-Stimulated RAW264.7 Cells.* Inflammation, 2017. **40**(2): p. 401-413.
26. Lin, R., et al., *13,14-seco-Withanolides from Physalis minima with Potential Anti-inflammatory Activity.* Chem Biodivers, 2016. **13**(7): p. 884-890.
27. Neurath, M., I. Fuss, and W. Strober, *TNBS-colitis.* Int Rev Immunol, 2000. **19**(1): p. 51-62.
28. Budhiraja, R., P. Krishan, and S. Sudhir, *Biological activity of withanolides.* Journal of Scientific and Industrial Research, 2000. **59**(11): p. 904-911.
29. Yu, H., et al., *The rat IgGFcgammaBP and Muc2 C-terminal domains and TFF3 in two intestinal mucus layers bind together by covalent interaction.* PLoS One, 2011. **6**(5): p. e20334.
30. Feng, Z.-M., et al., *Rodent IRR-219 (IgGFcγBP) and rTFF3, Expressed Mainly in the Intestinal Mucosa, Depleted During Dextran Sulfate Sodium–Induced Colitis.* Digestive Diseases and Sciences, 2007. **52**(9): p. 2104-2112.
31. Johansson, M.E., *Mucus layers in inflammatory bowel disease.* Inflamm Bowel Dis, 2014. **20**(11): p. 2124-31.
32. McCauley, H.A. and G. Guasch, *Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia.* Trends Mol Med, 2015. **21**(8): p. 492-503.
33. Pelaseyed, T., et al., *The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system.* Immunol Rev, 2014. **260**(1): p. 8-20.
34. Birchenough, G.M., et al., *New developments in goblet cell mucus secretion and function.* Mucosal Immunol, 2015. **8**(4): p. 712-9.
35. Ghosh, S. and M.S. Hayden, *Celebrating 25 years of NF-kB research.* Immunol Rev, 2012. **246**(1): p. 5-13.

36. Hausmann, M., et al., *Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation.* Gastroenterology, 2002. **122**(7): p. 1987-2000.
37. Kusugami, K., et al., *Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent.* Digestive Diseases and Sciences, 1995. **40**(5): p. 949-59.
38. Neurath, M.F., et al., *Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease.* Annals of the New York Academy of Sciences, 1998. **859**: p. 149-59.
39. Reinecker, H.C., et al., *Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease.* Clinical and Experimental Immunology, 1993. **94**(1): p. 174-81.
40. Bain, C.C. and A.M. Mowat, *Macrophages in intestinal homeostasis and inflammation.* Immunol Rev, 2014. **260**(1): p. 102-17.
41. Steinbach, E.C. and S.E. Plevy, *The role of macrophages and dendritic cells in the initiation of inflammation in IBD.* Inflamm Bowel Dis, 2014. **20**(1): p. 166-75.
42. Okabe, S., et al., *Disaccharide esters screened for inhibition of tumor necrosis factor-alpha release are new anti-cancer agents.* Jpn J Cancer Res, 1999. **90**(6): p. 669-76.
43. Li, W., et al., *NF-kB inhibitory activity of sucrose fatty acid esters and related constituents from Astragalus membranaceus.* J Agric Food Chem, 2013. **61**(29): p. 7081-8.

8 ACKNOWLEDGEMENTS

First and foremost, I want to thank my Lord Jesus Christ for refining me through this process. My doctoral training led me to the cross, put me on my knees and taught me how to lean on the loving hands of my Savior.

I want to give special thanks to my supervisor Luis Alberto Franco. I never imagined becoming a scientist until I met him. His advice changed my life completely, and for that I will always be in debt. I am sure that without his contribution in funding, time, ideas, and filial support, I would have never started my Ph.D. pursuit. It has been an honor to be your student!

I would also like to thank to my committee members, Professor Jesus Olivero and Professor Niradiz Reyes, as well as Professor Luis Caraballo, coordinator of the Ph.D. program, for expending their valuable time reviewing the project progress, and their excellent advices.

The members of the Biological Evaluation Group have contributed immensely to my professional and personal growth. You were a source of friendship, jokes, and collaboration. I am especially grateful to Daneiva Caro and Jenny Castro, for their scientific and spiritual support. I also thank to actual and former members of the group for their invaluable help during experiments. Finally, I greatly appreciate the collaboration from Professor Lia Barrios with the histology analysis.

The identification of peruvioses from *P. peruviana* discussed in Chapter 3 would not have been possible without the collaboration of Professor Jose Luis Espartero from the University of Seville, Spain. In this regard, I also offer a big thanks to Professor Harold Gomez for his help when preparing the manuscript to publish the isolation of peruvioses. I also give my sincere thanks to Professor Gerhard Rogler and my supervisors Isabelle Frey-Wagner and Kacper Wojtal for receiving me in their group at University Hospital Zurich. Finally, I can`t thank enough to Dr. Xuhang Li for inviting me to his lab at Johns Hopkins School of Medicine.

Last but not least I want to express my deep gratitude to my family. David, your love is a priceless gift from God that I intent to keep for the rest of my life. Thanks for providing me the support I needed and the tenderness of your heart. I also want to thank to my parents for encouraging me to take this path believing in my own skills more than I did, and patiently withstand my prolonged absences when experiments required so.

Finally, I want to express my gratitude to Colciencias [Grant 110751929179–2010 and 597-2012], the University of Cartagena [Grants 04670-2012, 055-2013 and Resolution 1446-2011], and Fulbright Colombia [Grant for Colombian PhD students to Yanet Ocampo] for the financial support to develop this project as well as my doctoral training.

9 APPENDIXES

Appendix 1. List of publications

a) Publications related to the doctoral thesis

Original Papers.

1. Luis Franco, **Yanet Ocampo**, Harold Gómez, Rocío De la Puerta, José Espartero, Luis Ospina: Sucrose esters from *Physalis peruviana* calyces with anti-Inflammatory activity. Planta Medica. v.80 issue.17 p.1605-1614, 2014.
   DOI: 10.1055/s-0034-1383192
   Impact Factor 2015: **1.99**. (**Q1**-Complementary and Alternative Medicine).

2. Jenny Castro*, **Yanet Ocampo***, Luis Franco: Cape Gooseberry [*Physalis peruviana* L.] Calyces Ameliorate TNBS Acid-induced Colitis in Rats. Journal of Crohn's and Colitis. v.9 issue.11 p.1004-1015, 2015.
   DOI: https://doi.org/10.1093/ecco-jcc/jjv132
   Impact Factor 2015: **6.585**. (**Q1**-Gastroenterology).
   *Both authors contributed equally as first authors.

3. **Yanet Ocampo**, Daneiva Caro, David Rivera, Luis Franco. Safety of sucrose esters from Physalis peruviana L. in a 28-day repeated-dose study in mice. Biomedicine & Pharmacotherapy Journal. 2017. Accepted for publication.
   Impact Factor 2015: **2.326**. (**Q2**-Pharmacology).

Scientific Events

1. **Yanet Ocampo**, Jenny Castro, Lía Barrios, David Rivera, Luis Franco, "Potential of Peruviose A and B from *Physalis peruviana* L calyces to treat Inflammatory Bowel Disease: *In vivo* and *in vitro* studies." 2013. 61st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Germany). GA-2013. POSTER. **DOI:** 10.1055/s-0033-1351979.

2. Jenny Castro, **Yanet Ocampo**, Luis Franco, "Anti-inflammatory intestinal activity of total ethereal extract from *Physalis peruviana* calyces in TNBS model of rat colitis". 2013. XX Congreso Latinoamericano de Farmacología y Terapéutica (Cuba). ORAL COMMUNICATION.

3. Elena Talero, Virginia Motilva, **Yanet Ocampo**, Luis Ospina, Luis Franco, "Anti-

inflammatory effect of the ether extract and major fraction of *Physalis peruviana* calyces in acute TNBS-induced colitis". 2012. Event: 6th European Congress of Pharmacology EPHAR 2012 (Spain). POSTER.
http://www.pa2online.org/abstracts/vol10issue3abst610p.pdf

4. Luis Franco, **Yanet Ocampo**, Harold Gómez, "Aislamiento, purificación e identificación de nuevos glicósidos de *Physalis peruviana* L." 2012. III Congreso de Química de Productos Naturales Chileno- Argentino-Hispano (Chile). ORAL COMMUNICATION.

5. Luis Franco, William Padilla, Vanessa Usta, Jenny Castro, Luis Ospina, **Yanet Ocampo**, "Extracto total etéreo de los cálices de *Physalis peruviana* L disminuye la inflamación intestinal en un modelo murino de colitis ulcerosa". 2012. XV Congreso de la Federación Farmacéutica Sudamericana FEFAS 2012 (Colombia). POSTER.

6. **Yanet Ocampo**, Indira Pájaro, Jenny Castro, Luis Ospina, Luis Franco, "Efecto protector de los cálices de *Physalis peruviana* L. En un modelo agudo de colitis ulcerosa en ratas." 2011. XI Congreso Colombiano de Fitoquímica (Colombia). POSTER.

Awards

1. **Award for the second best poster:** "Efecto protector de los cálices de *Physalis peruviana* L. En un modelo agudo de colitis ulcerosa en ratas." XI Congreso Colombiano de Fitoquímica (Colombia). October 2011.

2. **Colombian Doctoral Student Scholarship 2014.** Fulbright Colombia-Icetex. Medicine Trainee. Project "Potential of Sucrose Esters isolated from *Physalis peruviana* L to treat Inflammatory Bowel Disease". Johns Hopkins University School of Medicine. Gastrointestinal Division. Supervisor: Xuhang Li, PhD.

3. **PhD Scholarship through the National Program for Doctoral Formation** (Grant 597-2012). Colciencias.

b) Publications not related to the doctoral thesis

Besides the development of this doctoral thesis, my engagement as a doctoral student in the research group "Biological Evaluation of Promising Substances" required my participation in several research projects. As a result several original papers have been published in scientific journals.

Original Papers.

1. Sandra Coronado, Luis Barrios, Josefina Zakzuk, Ronald Regino, Velky Ahumada, Luis Franco, **Yanet Ocampo**, Luis Caraballo. A recombinant cystatin from *Ascaris lumbricoides* attenuates inflammation of DSS-induced colitis. Parasite Immunology. DOI: 10.1111/pim.12425
   Impact Factor: 1.917. (**Q2** - Immunology/Parasitology)

2. Caro D, **Ocampo Y**, Castro J, Barrios L, Salas R, Franco LA. Protective effect of *Dracontium dubium* against *Bothrops asper* venom. Biomed Pharmacother. 2017 89:1105-1114.
   DOI: 10.1016/j.biopha.2017.02.080.
   Impact Factor 2015: 2.326. (**Q2** - Pharmacology).

3. Rivera D, **Ocampo Y**, Castro J, Caro D, Franco L. Antibacterial activity of *Physalis angulata* L., *Merremia umbellata* L., and *Cryptostegia grandiflora* Roxb. Ex R.Br.- medicinal plants of the Colombian Northern Coast. Oriental Pharmacy and Experimental Medicine. 2015; 15:1-8.
   DOI: 10.1007/s13596-014-0176-0
   Quartiles: (**Q2** - Complementary and Alternative Medicine)

4. Castro J, **Ocampo Y**, Franco L. *In vivo* and *in vitro* anti-inflammatory activity of *Cryptostegia grandiflora* Roxb. ex R. Br. leaves. Biological Research. 2014; 47(32):1-8.
   DOI: 10.1186/0717-6287-47-32.
   Impact Factor: 1.328. (**Q1** - Agricultural and Biological Sciences-Miscellaneous)

5. Franco L, **Ocampo Y**, Gaitan R. Nitric oxide inhibitory activity of hydrogenated synthetic analogues of furanonaphthoquinones isolated from *Tabebuia* spp. Revista Cubana de Farmacia. 2013; 47(4):502-516.
   Quartiles: (**Q3** - Pharmacy)

Appendix 2. IR, FAB-MS, ^{1}H-NMR, and ^{13}C-NMR (Dep90-Dep135) spectra obtained for peruvioses A and B mixture.

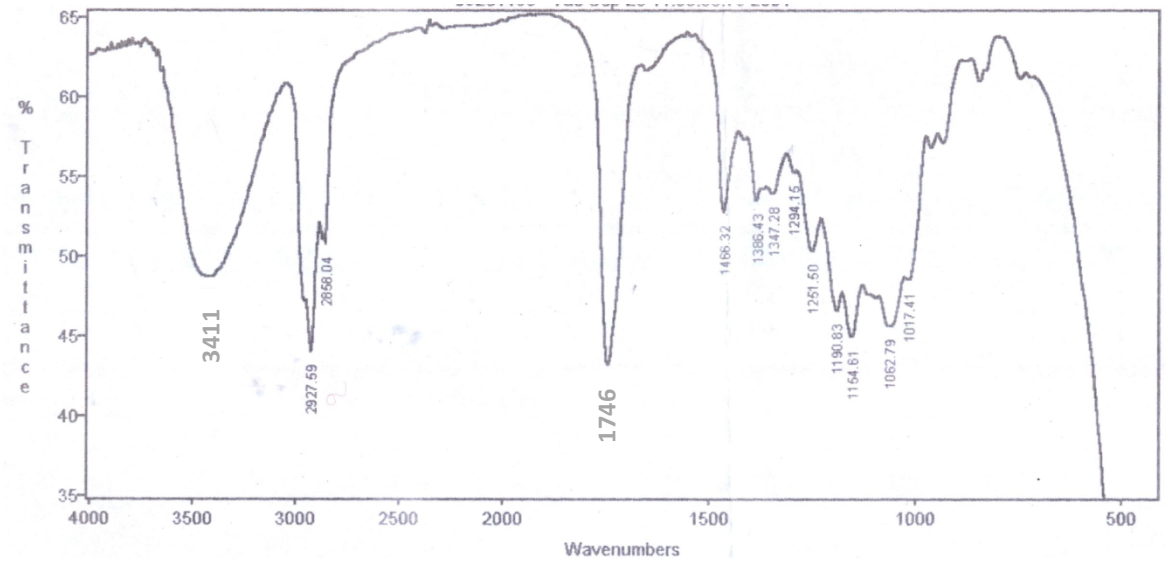


Figure A1. IR spectrum of the mixture of peruvioses A **(1)** and B **(2)**.

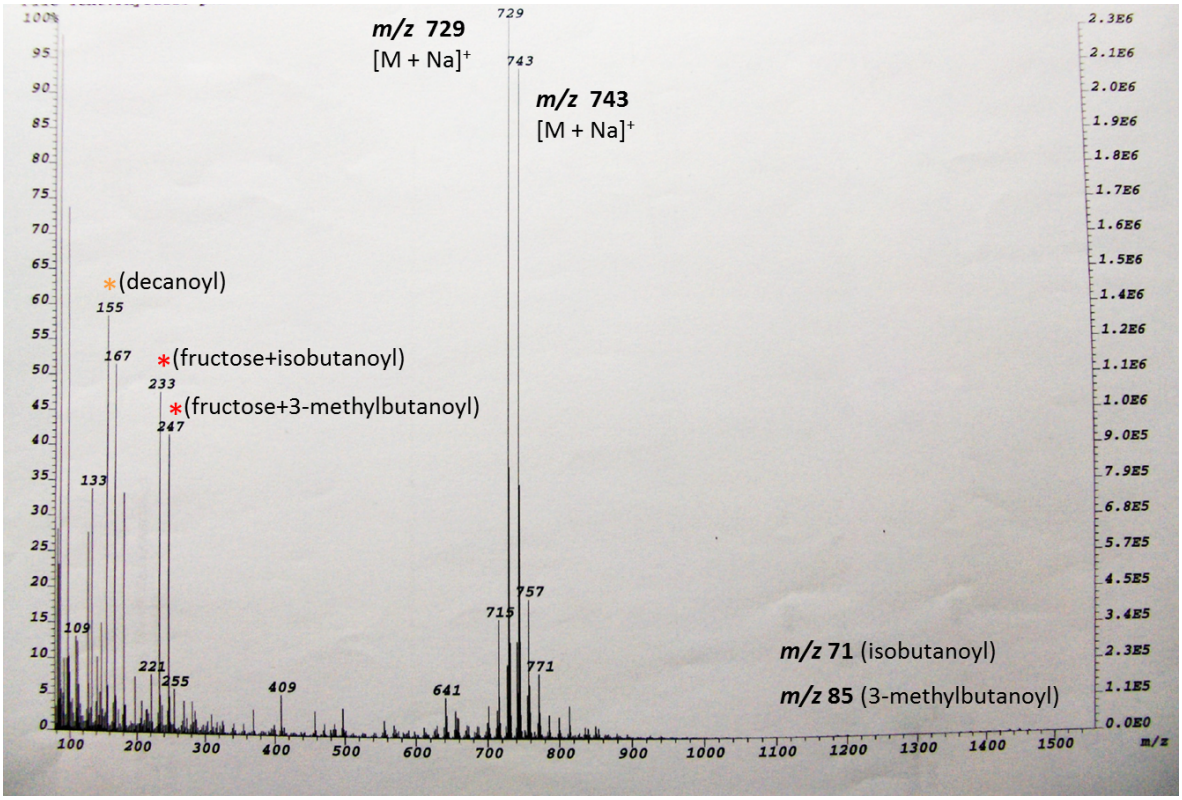


Figure A2. FAB mass spectrum of the mixture of peruvioses A **(1)** and B **(2)**.

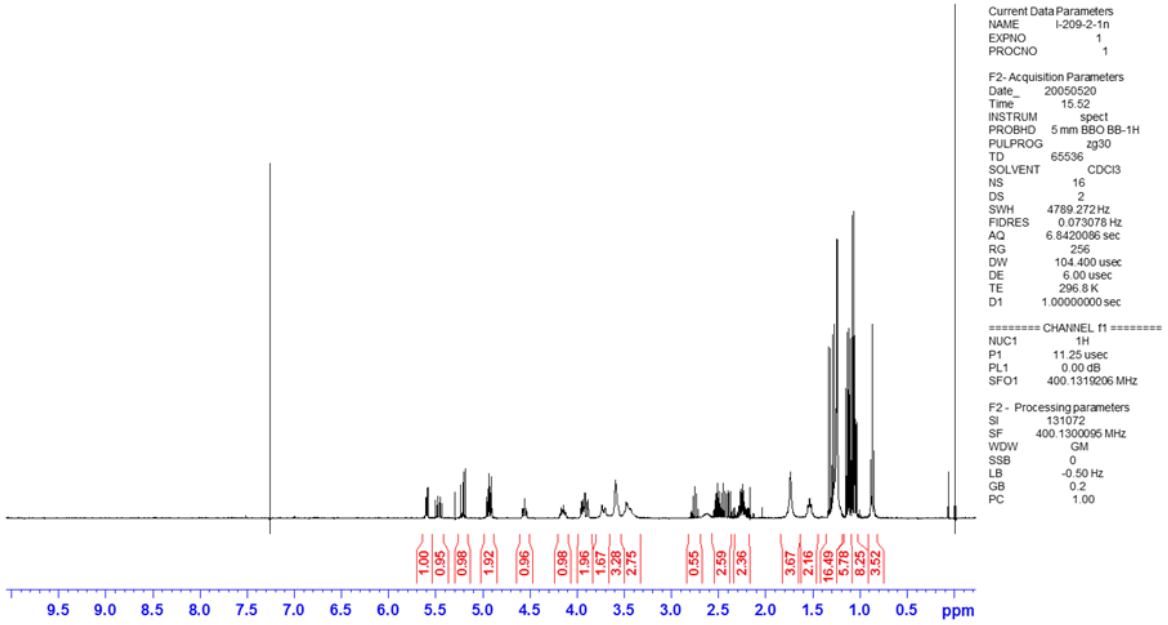


Figure A3. ^{1}H NMR spectrum of the mixture of peruviose A **(1)** and B **(2)** in $CDCl_3$ (500 MHz).

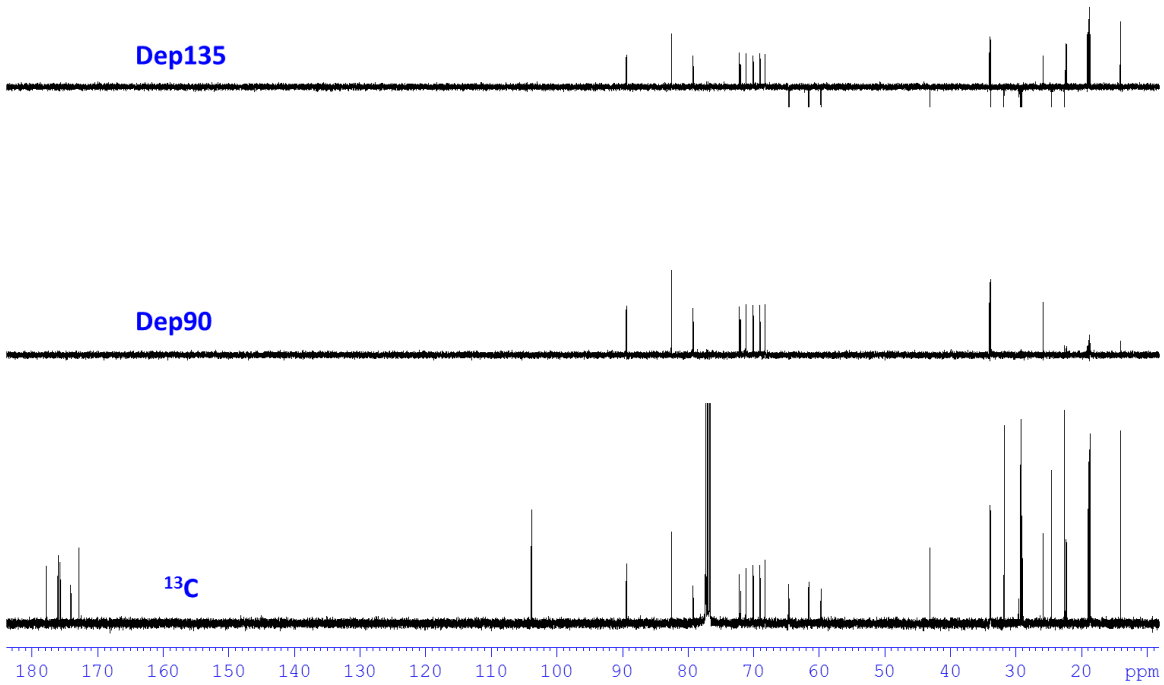


Figure A4. ^{13}C NMR spectrum of the mixture of peruviose A **(1)** and B **(2)** in $CDCl_3$ (125 MHz)

Appendix 3. Proof of Approval by the Ethics Committee of the University of Cartagena

The attached document certifies that the Ethics Committee from the University of Cartagena approved the performance of the project "Identificación de las dianas moleculares implicadas en el efecto anti-inflamatorio e inmunomodulador producido por compuestos aislados de *Physalis peruviana* L., en un modelo de colitis ulcerosa" presented by Luis Franco Ospina, as stated in the minutes of October 14 of 2010.

Universidad de Cartagena
Vice-Rectoría de Investigaciones

EL PRESIDENTE DEL COMITÉ DE ÉTICA EN INVESTIGACIONES DE LA UNIVERSIDAD DE CARTAGENA

CERTIFICA QUE:

El proyecto titulado **"IDENTIFICACIÓN DE LAS DIANAS MOLECULARES IMPLICADAS EN EL EFECTO ANTI-INFLAMATORIO E INMUNOMODULADOR PRODUCIDO POR COMPUESTOS AISLADOS DE *PHYSALIS PERUVIANA* L., EN UN MODELO DE COLITIS ULCEROSA"**, presentado por **LUIS ALBERTO FRANCO OSPINA**, docente investigador de la Facultad de Ciencias Farmacéuticas, de la Universidad de Cartagena, no presenta impedimentos éticos, de acuerdo a lo contemplado en la Resolución 8430 de 1993 del Ministerio de Salud y en el Reglamento de Ética de la Universidad de Cartagena, tal como consta en el Acta N° 23 del Comité de Etica en Investigaciones del 14 de octubre de 2010.

Para constancia se firma en la Ciudad de Cartagena, a los catorce (14) días del mes de octubre del año dos mil diez (2010).

ALVARO OLIVERA DIAZ, MD
Presidente

¡Siempre a la altura de los tiempos!
Sede Claustro de la Merced. Centro. Plaza de la Merced, Cra. 4 N° 38-40, Segundo Piso. Telefax 6642663
E-mail: vicerectoriadeinvestigaciones@gmail.com - investigaciones@unicartagena.edu.co
Cartagena de Indias, D. T. H. y C.

Appendix 4. Histological Analysis

As described in Material and Methods, slides from colon, liver, and kidney from rodents were coded, stained with Hematoxylin & Eosin, and evaluated in a blinded manner by a pathologist. In the case of sections from spleen, heart, ovaries, and testes, the evaluation was performed without a scoring system.

Table A1. Scoring Criteria of Colon, Liver and Kidney Sections.

COLON [a]	LIVER [b]	KIDNEY [b]
Mucosa	**Inflammation and Necrosis/Apoptosis**	Edema
Ulceration	Portal Space	Interstitial inflammatory cell infiltration
PMN infiltration	Mild Zone	Tubulitis
Mononuclear cells infiltration	Lobular Zone	Acute tubular necrosis
Edema and dilation of lacteals	**Hepatocellular Damage**	Distalization of Tubules
Crypt: Mitotic activity	Ballooning degeneration	Fibrosis
Crypt: Dilations	Vacuolization	Regenerative Changes
Crypt: Goblet cell depletion	Cholestasis	Vascular Congestion
Submucosa	**Regenerative Changes**	
PMN infiltration	**Vascular Congestion**	
Mononuclear cells infiltration	**Neoplasia**	
Edema		
Muscular		
PMN infiltration		
Mononuclear cells infiltration		
Serosa		
Infiltration		

a. Scoring scale: 0, none; 1, slight; 2, moderate; 3, severe; 4, extensive. Maximum score: 52 (Colon).

b. Scoring scale: 0, none; 1, slight; 2, moderate; 3, severe. Maximum score: 27 (Liver) and 24 (Kidney).

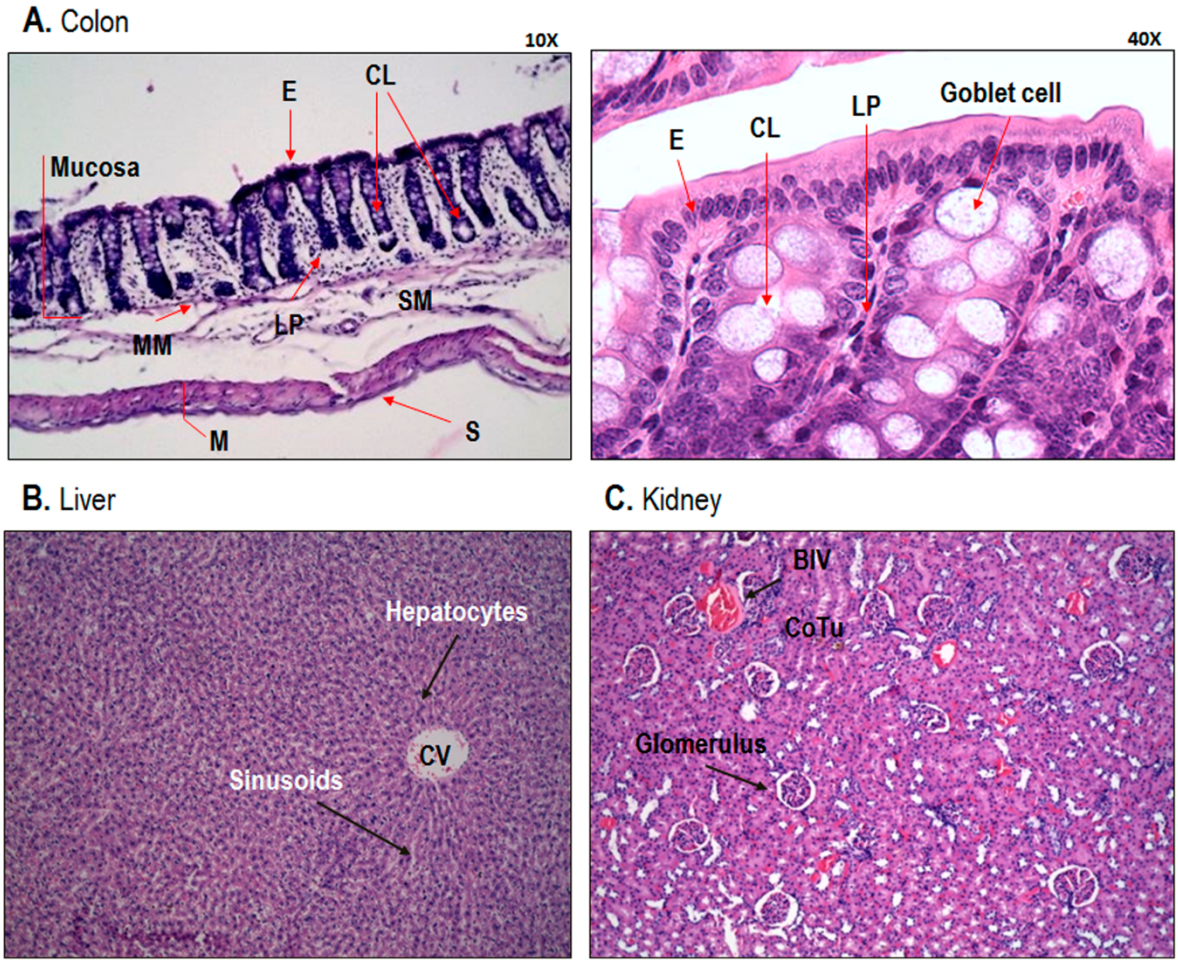


Figure A5. Overview of the histological appearance of rat colon, liver and kidney sections stained with hematoxylin & eosin (H&E). Representative pictures showing: **(A)** Normal colon architecture that is formed by, from inside to outside, mucosa, a poorly developed muscularis mucosae (MM), submucosa (SM), muscularis externa (M), and serosa (S). The mucosa of the large intestine, comprising simple columnar epithelium (E) and a lamina propria (LP), forms deep cavities, the crypts of Lieberkühn (CL). The epithelium contains absorptive enterocytes with apical microvilli and many oval, mucin producing goblet cells. **(B)** Normal liver architecture, and components of basic liver lobules, with central vein (CV), sinusoids and sheets of hepatocytes in the liver parenchyma. **(C)** Normal kidney cortex which displays numerous glomeruli and the convoluted tubes (CoTu), as well as blood vessel (BlV). Original magnification 10X (colon, liver, and kidney) and (40X, colon).

Appendix 5. Real Time PCR Analysis

Table A2. Sequences of Primers used for Real-time PCR analysis with rat tissue.

Gene Symbol	Gene ID	Official Name	Primer sequences [a]	Amplicon Size (pb)
iNOS	24599	Nitric oxide synthase 2	F: CAGCCCTCAGAGTACAACGAT R: CAGCAGGCACACGCAATGAT	91
COX-2	29527	Prostaglandin-endoperoxide synthase 2	F: ATCAGGTCATCGGTGGAGAG R: CTGCTTGTACAGCGATTGGA	579
TNF-α	24835	Tumor necrosis factor alpha	F: ATGTGGAACTGGCAGAGGAG R: AGTAGACAGAAGAGCGTGGTG	127
			F: AACACACGAGACGCTGAAG R: AAGGAAGGAAGGAAGGGAGG	207
IL-1β	24494	Interleukin 1 beta	F: CTCCATGAGCTTTGTACAAG R: TGCTGATGTACCAGTTGGGG	245
IL-6	24498	Interleukin 6	F:TCCTACCCCAACTTCCAATGCTC R: TTGGATGGTCTTGGTCCTTAGCC	79
IL-10	25325	Interleukin 10	F: CCAGTCAGCCAGACCCACAT R: TGGCAACCCAAGTAACCCT	143
IL-17A	301289	Interleukin 17A	F: TGGACTCTGAGCCGCAATGA R: GACGCATGGCGGACAATAGA	245
MUC-2	24572	Mucin 2	F: GTGGCATCAACTTTGTGGTG R: TGTTGCCAAACAGGTGGTAA	102
MUC-3	687030	Mucin 3	F: GGCTCAAGGACAATAAGTGGTA R: ATCCGTGGACAAGCAGAGG	128
TFF3	25563	Trefoil factor 3	F: GCCTATCTCCAAGCCAATGTAT R: TGAAGCACCAGGGCACAT	135
NF-κB	309165	Nuclear Factor-kappa B	F: ACCTGGAGCAAGCCATTAGC R: CGGACCGCATTCAAGTCATA	100
HPRT	24465	Hypoxanthine phosphoribosyltransferase 1	F: TCCCAGCGTCGTGATTAGTGA R: CCTTCATGACATCTCGAGCAAG	152
GAPDH	24383	Glyceraldehyde-3-phosphate dehydrogenase	F: GCTGGTCATCAACGGGAAA R: ACGCCAGTAGACTCCACGACA	105

[a] F: Forward (5'→3'); R: Reverse (5'→3').

Table A3. Sequences of Primers used for Real-time PCR analysis with mouse tissue.

Gene Symbol	Gene ID	Official Name	Primer sequences [a]	Amplicon Size (pb)
Cd36	12491	CD36 antigen	F: GGAGCAACTGGTGGATGGTT R: CTACGTGGCCCGGTTCTAAT	145
Fasn	14104	Fatty acid synthase	F: TCCACCTTTAAGTTGCCCTG R: TCTGCTCTCGTCATGTCACC	185
Lpl	16956	Lipoprotein lipase	F: TGCCGCTGTTTTGTTTTACC R: TCACAGTTTCTGCTCCCAGC	164
Ppara	19013	Peroxisome proliferator activated receptor alpha	F: GGGTACCACTACGGAGTTCACG R: CAGACAGGCACTTGTGAAAACG	160
Scd1	20249	Stearoyl-Coenzyme A desaturase 1	F: TTCTTGCGATACACTCTGGTGC R: CGGGATTGAATGTTCTTGTCGT	98
Srebf1	20787	Sterol regulatory element binding transcription factor 1	F: CAAGGCCATCGACTACATCCG R: CACCACTTCGGGTTTCATGC	61
Gapdh	14433	Glyceraldehyde-3-phosphate dehydrogenase	F: CATGGCCTTCCGTGTTCCT R: AGTTGGGATAGGGCCTCTCTTG	378

[a] F: Forward (5'→3'); R: Reverse (5'→3'). [b] Tm: Melting Temperature.