Anti-inflammatory properties of an extract of M. ilicifolia in the human intestinal epithelial Caco-2 cell line

Wonfor, Ruth; Natoli, Manuela; Shah, Ifat; Beckmann, Manfred; Nash, Robert J.; Nash, Deborah

Published in:
Journal of Ethnopharmacology

DOI:
10.1016/j.jep.2017.08.006

Publication date:
2017

Citation for published version (APA):
Wonfor, R., Natoli, M., Shah, I., Beckmann, M., Nash, R. J., & Nash, D. (2017). Anti-inflammatory properties of an extract of *M. ilicifolia* in the human intestinal epithelial Caco-2 cell line. *Journal of Ethnopharmacology*, *209*, 283-287. https://doi.org/10.1016/j.jep.2017.08.006

Anti-inflammatory properties of an extract of *M. ilicifolia* in the human intestinal epithelial Caco-2 cell line

Ruth Wonfor, Manuela Natoli, Ifat Parveen, Manfred Beckman, Robert Nash, Deborah Nash

www.elsevier.com/locate/jep

PII: S0378-8741(17)31236-9
DOI: http://dx.doi.org/10.1016/j.jep.2017.08.006
Reference: JEP10976

To appear in: *Journal of Ethnopharmacology*

Received date: 29 March 2017
Revised date: 31 July 2017
Accepted date: 2 August 2017

Cite this article as: Ruth Wonfor, Manuela Natoli, Ifat Parveen, Manfrec Beckman, Robert Nash and Deborah Nash, Anti-inflammatory properties of a extract of *M. ilicifolia* in the human intestinal epithelial Caco-2 cell line, *Journa of Ethnopharmacology,* http://dx.doi.org/10.1016/j.jep.2017.08.006

Anti-inflammatory properties of an extract of *M. ilicifolia* in the human intestinal epithelial Caco-2 cell line

Ruth Wonfor[a1], Manuela Natoli[a12], Ifat Parveen[a], Manfred Beckman[a], Robert Nash[b], Deborah Nash[a*]

[a]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Penglais, Aberystwyth, Ceredigion, SY23 3DA, UK.

[b]Phytoquest Ltd, Plas Gogerddan, Aberystwyth, Ceredigion, SY23 3EB, UK.

*Corresponding author. dmn@aber.ac.uk (D. Nash). Tel.: +44 (0) 1970 622575.

rec21@aber.ac.uk

manuela.natoli@gmail.com

ifp@aber.ac.uk

meb@aber.ac.uk

robert.nash@phytoquest.co.uk

dmn@aber.ac.uk

Abstract

Ethnopharmacology relevance

Maytenus ilicifolia is a Celastracea plant used in traditional medicine to alleviate digestive tract inflammatory disorders.

Aim of the study

We investigated anti-inflammatory properties of *M. ilicifolia* crude extract towards Caco-2 cell line, as a model of Toll-like Receptor 2 (TLR-2) inflammatory pathway.

Materials and Methods

[1] These authors contributed equally to this work.

[2] M. Natoli is now based at Cancer Research UK, 42 Regent Street, Cambridge, CB2 1DP, UK.

Toxicity was assessed following culture of Caco-2 with *M. ilicifolia*, using apparent cell permeability and trans-epithelial electric resistance. Anti-inflammatory properties of *M. ilicifolia* were assessed through IL-8 secretion and TLR-2 associated gene expression of Caco-2 cells with or without an LTA challenge.

Results

M. ilicifolia was not toxic to Caco-2 cells. *M. ilicifolia* down-regulated TLR2 expression with and without LTA challenge but had no effect on other genes. Following LTA challenge of Caco-2 cells, 100 and 200µg/mL *M. ilicifolia* abrogated IL-8 secretion.

Conclusions

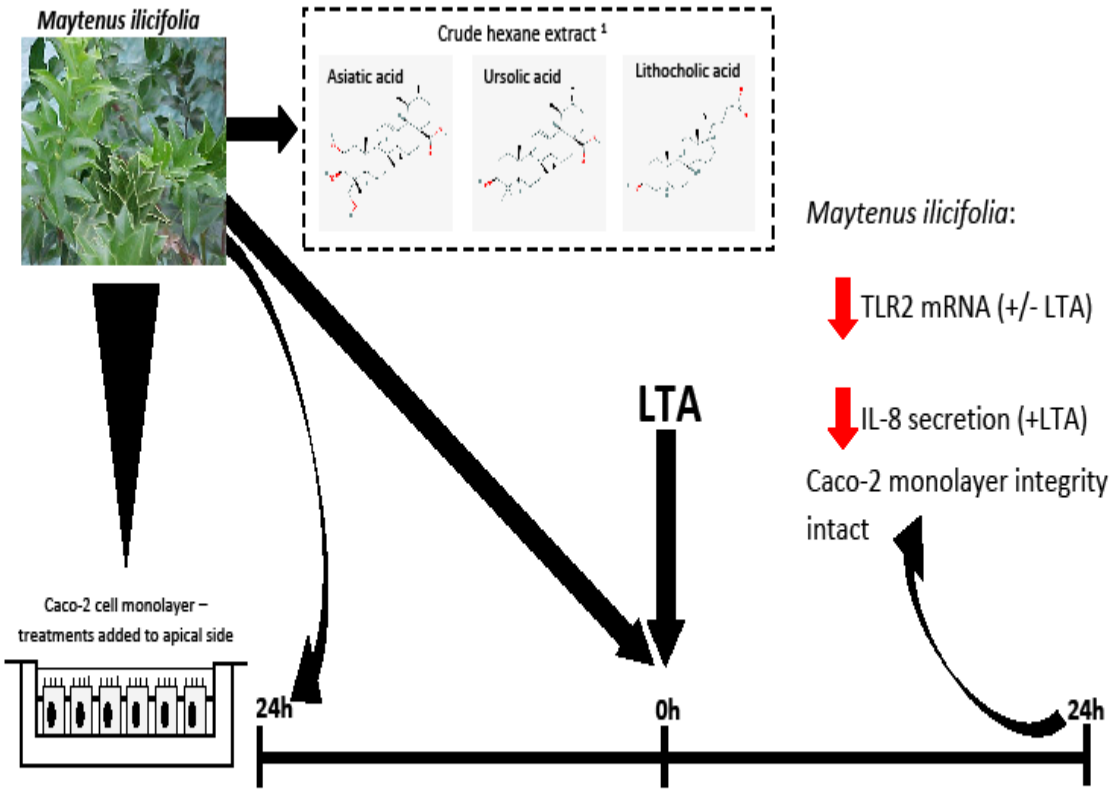
We provide preliminary data for some *M. ilicifolia* anti-inflammatory properties. Further research must establish the full extent and mode of action on particular inflammatory pathways.

List of abbreviations

IL-8, Interleukin-8; LTA, Lipoteichoic acid; PAPP, Phenol red apparent permeability; TEER, Trans-epithelial electrical resistance; TLR-2, Toll-like receptor-2

Graphical abstract

2d chemical structures for hexane extract compounds retrieved from: National Center for Biotechnology Information. PubChem Compound Database https://pubchem.ncbi.nlm.nih.gov/compound/ (accessed Mar. 7, 2017).



Keywords: Inflammation. Caco-2 cells. *M. ilicifolia*. Toxicity. Anti-inflammatory

1. Introduction

Celastracea is a family of plants including the genus, *Maytenus* (Born, 2000). *Maytenus ilicifolia*, native to Brazil, is used in traditional medicine to alleviate digestive inflammatory disorders, such as ulcers (Cipriani et al., 2006; Leite et al., 2001). There are widespread anecdotal reports of the anti-ulcerative and anti-inflammatory properties of *M. ilicifolia* (Born, 2000). *M. ilicifolia* crude extract reduces induced paw oedema in rodents (Jorge et al., 2004), however, there are no data for anti-inflammatory effects towards cells of the digestive tract, *in vivo* or *in vitro*.

The human intestinal epithelial cell (IEC) line, Caco-2, exhibits a measurable inflammatory response via Toll-like receptor-2 (TLR-2), when stimulated by bacterial ligands (Kang et al., 2015; Laparra, 2013). Expression of cytokines downstream of TLR-2 activation (Tumour necrosis factor-α, *TNF*; Interleukin-1β, *IL1B* and Interleukin-8, *CXCL8*) (Dziarski and Gupta, 2000) serve as biomarkers of inflammation, as well as factors such as heat shock protein 70 (*HSPA1A*) which modulates TLR-2 and acts as a cytokine itself (Asea et al., 2000; Asea et al., 2002). We established that Caco-2 cells secrete Interleukin-8 (IL-8) in a dose-dependent inflammatory response induced by lipoteichoic acid (LTA; data not presented). In terms of inflammation, one use of the Caco-2 cell line is to investigate models of inflammatory bowel disease and the inflammatory responses of the IEC in this context (Fernandes et al., 2016; Perey et al., 2015); the pathogenesis of this disease has underlined our choices of inflammatory mediators investigated.

This preliminary study determined the anti-inflammatory activity of a crude extract of *M. ilicifolia* using Caco-2 cells by 1) determination of toxicity; 2) characterisation of the effect of *M. ilicifolia* on expression of genes associated with TLR-2 pathway; 3) assessment of anti-inflammatory activity of *M. ilicifolia* using Caco-2 cells after challenge with LTA by measuring IL-8 secretion.

2. Materials and methods

2.1 M. ilicifolia plant collection

Maytenus ilicifolia Mart. Ex. Reiss., (Celastraceae) source plant material was collected in July 2013 from the municipality of Irati, State of Paraná, Brazil.

2.1 M. ilicifolia characterisation and extract preparation

Maytenus ilicifolia Mart. Ex. Reiss., (Celastraceae) source plant material was formally identified. A herbarium voucher specimen has been kept at IBERS (R. Nash 102016). An extract from dried ground *M. ilicifolia* leaves was prepared at Bangor University (Dr Vera Thoss and Dr Marc Bouillon) using hexane.

2.2 Extract analysis

20μL of sample were injected onto a Waters C_{18} reverse-phase Nova-Pak column (4.0μm, 3.9mm × 100mm) and run on the Thermo Finnigan HPLC/MS^n system (Thermo Electron Corporation) as previously reported (Parveen et al., 2013). The initial mobile phase was water (95%) and MeOH / HCO_2H (1000:1) (5%), changing linearly to MeOH / HCO_2H (1000:1) (5%) during 30 min.

Ultra-high accurate mass analysis was performed using a nano-flow linear trap quadrupole Fourier Transformation Ion Cyclotron Resonance Mass Spectrometry Ultra (FT-ICR-MS) (Triversa Nanomate; Advion Biosciences Ltd.). Sample was diluted with MeOH/ultra-pure water (7:3; 100μL) and centrifuged for 2 minutes, at 13,000rpm at 0ºC. 10μL samples were injected by the injection system, with 5μL delivered to the ICR cell.

2.3 Caco-2 cell culture

Frozen Caco-2 cells from passage 100 (from Dr D'Agnano; CNR-IMCB Monterotondo, Rome) were thawed and maintained in supplemented, complete DMEM at 37°C, 10% CO_2. At 50% confluence, cells were passaged using trypsin-EDTA and maintained as described (Natoli et al., 2012). Cells were seeded on PET transwell membrane inserts (4.71 cm^2) at a density of 3×10^5 cm^{-2} and maintained for 21 days in complete medium by which time differentiation occurred.

2.4 Experimental design

A preliminary experiment titrated a range of ultrapure LTA (*Staphylococcus aureus*; Invivogen) concentrations in the range 1-50μg/mL to determine the concentration used in the present study (15μg/mL; data not shown).

Cells were cultured in medium alone (control), or medium with *M. ilicifolia* extract (12.5, 25, 50, 100 or 200μg/mL) for 24h pre-treatment. Medium was then removed and replaced with fresh medium alone (control) or containing the same concentrations of *M. ilicifolia* with or without LTA. All treatments were added to the apical compartment. Plates were incubated for a further 24h, after which cells and medium were harvested for gene expression or IL-8 secretion analysis, respectively.

Each experiment was repeated using three separate passages and experiments conducted in duplicate. Cells were lysed in lysis buffer and stored at -80°C and supernatants at -20°C.

2.5 Toxicity tests

The effect of *M. ilicifolia* on the epithelial integrity of the Caco-2 cell monolayer with or without LTA was determined using the same concentrations. The integrity of the differentiated monolayer was determined by measuring the trans-epithelial electric resistance (TEER) using a commercial apparatus (Millipore). Final values were expressed as $\Omega*cm^2$. Phenol red apparent permeability (PAPP) was measured as described (Ferruzza et al., 2003).

2.6 Gene expression

Total RNA was purified using the Total RNA Purification Plus Kit (Norgen), quantified and quality estimated using a NanoDrop ND-1000. cDNA was synthesised using First Strand cDNA Synthesis Kit (Thermo Scientific) and subjected to real-time PCR analysis (Bio-Rad thermal cycler) with Maxima SYBR green/ROX Master Mix (Thermo Scientific). Primers (Supplementary Table 1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi).

2.7 Protein secretion

IL-8 concentrations in culture medium was quantified using a DuoSet ELISA kit (R&D), performed according to manufacturer instructions. The optical density was read unstopped with an iMark microplate reader (Bio-Rad) at 450nm.

2.8 Statistical analysis

Data analyses were performed using SPSS (version 22). Gene expression data were normalised using the Cq values of the internal controls *PPIA* and *TBP*. IL-8 concentrations were reported as the arithmetic mean±SEM. ANOVA was used to compare TEER, PAPP, IL-8 secretion (with Dunnet's post hoc) or gene-expression fold-induction (using a least significance difference for

mRNA expression data analysis), between control and treated cells. Statistical significance was assumed if $P<0.05$.

3. Results

Three isomers of asiatic acid in the hexane tissue extract were detected; 487 [M - 1] and less intense ions *m/z* 469 [M - H_2O] and 443 [M – CO_2] in negative ion mode and ions *m/z* 512 [M + Na], 489 [M + H] and 471 [M + H – H_2O] in positive-ion mode. Other terpenes were detected, including ursolic acid and lithocholic acid.

The TEER of Caco-2 cells was reduced by all *M. ilicifolia* concentrations ($P<0.05$) compared to the untreated control, but not in the presence of LTA (Supplementary Figure 1a). However, *M. ilicifolia* did not affect PAPP of the differentiated monolayer of Caco-2 cells, with or without LTA stimulation (Supplementary Figure 1b) demonstrating cell monolayer integrity.

Expression of *TLR2* in Caco-2 cells was diminished in a dose-dependent manner by the *M. ilicifolia* extract compared to untreated controls (Figure 1a). For cells without LTA, compared to the untreated control, 50μg/mL extract tended to reduce *TLR2* expression ($P<0.08$) and 100 and 200μg/mL extract reduced *TLR2* expression ($P<0.05$ and $P<0.01$, respectively). For cells treated with LTA, compared to the untreated control, 100μg/mL *M. ilicifolia* tended to reduce *TLR2* expression ($P<0.08$) and 200μg/mL extract reduced *TLR2* expression ($P<0.001$).

Caco-2 cells treated with 200μg/mL extract alone demonstrated increased expression of the *CXCL8* gene ($P<0.05$), compared to untreated control (Figure 1b). No other genes were affected by *M. ilicifolia*, with or without LTA (Figure 1c, d, e, f).

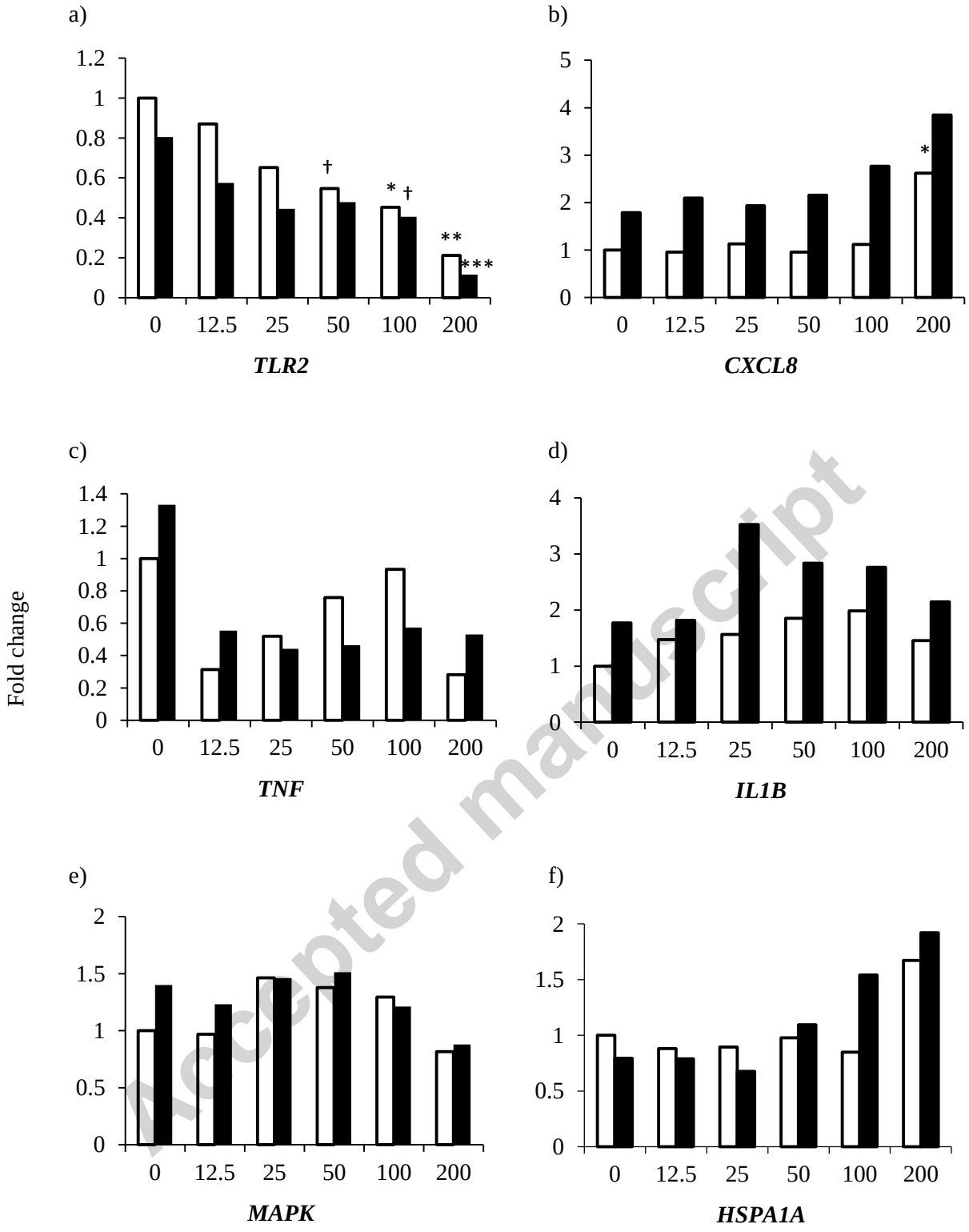
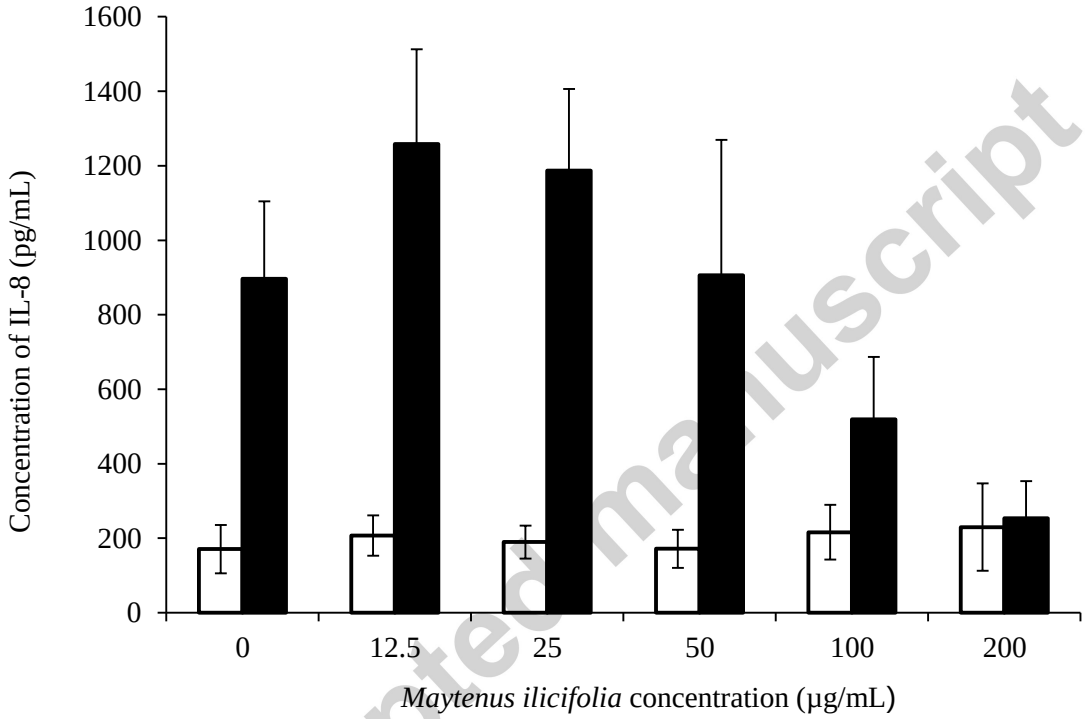


Figure 1: *Maytenus ilicifolia* modulates genes associated with the TLR-2 inflammatory pathway expressed by Caco-2 cells (□) and with 15µg/mL LTA (■): a) *TLR2*, b) *CXCL8*, c) *TNF*, d) *IL1B*, e)

MAPK and f) *HSPA1A*. Fold change was significantly different to the control: $^{\dagger}P<0.08$ (tendency); *P<0.05; **P<0.01; ***P<0.001.

Cells treated with LTA in combination with 0, 12.5, 25 or 50μg/mL extract, secreted increased IL-8 concentrations, compared to the 0 control (*P<0.05, **P<0.01, **P<0.01, *P<0.05, respectively; Figure 2). However, there was no difference in IL-8 secretion between cells treated with LTA in combination with 100 or 200μg/mL *M. ilicifolia* extract, compared to the 0 control. Therefore, *M. ilicifolia* extract abrogated the secretion of IL-8 in the presence of an inflammatory challenge (Figure 2).



*

Figure 2: Secretion of IL-8 from differentiated CaCo-2 cells cultured in medium alone (control), with *Maytenus ilicifolia* (☐) or with *Maytenus ilicifolia* in the presence of 15μg/ml LTA (■). Concentration of IL-8 secretion was greater than control: * P<0.05; ** P<0.01.

4. Discussion

Terpenes were obtained from the hexane extract of *M. ilicifolia*. Three isomers of asiatic acid were identified. Ions *m/z* 511 [M + Na], 489 [M + H] and 471 [M + H – H_2O] were detected and fragmentations were typical of those previously reported (Gajbhiye et al., 2016). Similar fragmentation patterns were detected for the other isolated terpenes, *e.g.* ursolic acid and lithocholic

acid. Triterpenes such as asiatic acid are reported to have anti-inflammatory activity within human colon cancer cells, as well as LPS-induced macrophages or lung tissue (Li et al., 2016; Yang et al., 2014; Yun et al., 2008). In agreement with the present study, triterpenes have been identified as a component of *M. ilicifolia,* in a hexane crude extract, which reduced gastric lesions in rats (Jorge et al., 2004).

M. ilicifolia was not toxic to the Caco-2 cell line, demonstrated through integrity of the cell monolayer. Thus, the *M. ilicifolia* extract was deemed appropriate to use, at the concentrations tested for toxicity, to assess any anti-inflammatory actions using this *in vitro* Caco-2 cell model.

Of the genes assessed, only *TLR2* expression was downregulated by *M. ilicifolia* extract (200μg/mL), compared to untreated cells, in the presence and absence of LTA. This is the first study to investigate the effects of *M. ilicifolia* on *TLR2* expression and any mechanistic explanation would be speculative until further study. As an exemplar of intestinal inflammation, in the pathogenesis of inflammatory bowel disease, TLRs are acknowledged to be important (Walsh et al., 2013), yet the role of TLR-2 is, at present, controversial. Some studies outline an upregulation of TLR-2 protein and gene expression in inflamed intestinal mucosa (Hausmann et al., 2002; Szebeni et al., 2008), whilst others demonstrate no differential gene expression of *TLR2* (Fernandes et al., 2016). Yet, it is clear that TLRs act as pathogen recognition receptors, and TLR-2 specifically detects LTA, amongst other ligands, which leads to activation of the downstream pathway, resulting in secretion of cytokines such as IL-8. Thus, the presence of LTA on bacteria such as *S. aureus* in the intestine will increase secretion of IL-8 from IEC. Therefore, by reducing the gene expression of *TLR2* it is possible that *M. ilicifolia* also reduces the protein expression of TLR-2 and thus reduces the active sites for stimulation of this particular pathogen recognition recetor. However, further study is necessary to quantify the protein expression of TLR-2 on Caco-2 cells in response to *M. ilicifolia*. It would be of interest to establish the effect of *M. ilicifolia* on other TLR implicated in inflammatory bowel disease, such as TLR-4 (Fernandes et al., 2016; Walsh et al., 2013).

For Caco-2 cells challenged with LTA, *M. ilicifolia* (100 and 200μg/mL) abrogated secretion of the potent chemokine IL-8, returning concentrations close to control levels, providing the first preliminary evidence for anti-inflammatory effects of *M. ilicifolia*. However, IL-8 equivalent *CXCL8* gene expression did not change which may suggest a post-translational mechanism. Protein secretion is an important indicator of anti-inflammatory activity and these data provide evidence. IL-8 is a chemokine of interest in relation to IEC inflammatory response studies, indicated by its role in inflammatory bowel disease (Kaser et al., 2010) following stimulation by circulating factors linked to the disease, such as TNF-α (Jung et al., 1995; Perey et al., 2015). As inflammatory bowel disease can be further complicated by the presence of *S. aureus* (Bettenworth et al., 2013), Kang et al. (2015) began to elucidate the interaction of *S. aureus* with IEC. They demonstrated that *S. aureus* induced an increase of gene expression and protein secretion of IL-8, a moderate increase in *IL1B* expression but no change in *TNF* or *IL6* from Caco-2 cells (Kang et al., 2015). Therefore, this preliminary study focussed on secretion of IL-8 due to the importance of its secretion from IEC in inflammatory bowel disease. Yet there are several other cytokines of importance secreted from IEC in cases of inflammatory bowel disease, such as IL-10, which we will investigate in future studies.

The effect of *M. ilicifolia* on *TLR2* expression and abrogation of IL-8 secretion, following an inflammatory challenge, demonstrates some anti-inflammatory ability. This is consistent with Jorge et al. (2004), where anti-inflammatory effects were measured in terms of paw lesions and oedema in mice or using anti-ulcerative experiments in mice and rats. This is the first study to report potential therapeutic properties of *M. ilicifolia* using Caco-2 cells, which is directly and biologically relevant to human duodenal and gastrointestinal disorders. However, a wider range of immune markers and time points of assessment for both gene and protein expression must be conducted to fully characterise the mechanisms and scope of *M. ilicifolia* anti-inflammatory functionality.

Acknowledgments

We thank Dr Carol Michael for supplying freeze-dried *M. ilicifolia* for use in this study. This work was funded by a Technology Strategy Board Innovation Voucher. The authors gratefully acknowledge support from Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant on Energy Grasses and Biorefining (BB/J0042/1) and BEACON from the European Regional Development Fund through the Welsh European Funding Office, part of the Welsh Assembly.

Authors' contributions

RW and MN designed the cell-based experiments, conducted that laboratory work and proof read the manuscript. IPF and MB performed HPLC to characterise the *M. ilicifolia* crude extract constituents. RJN identified the source material. RJN and IFP wrote paragraphs in the manuscript relating to identification and characterisation of *M. ilicifolia* respectively. DN initiated the project, secured funding, analysed the data and wrote the remainder of the manuscript.

References

Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C., Calderwood, S.K., 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nature medicine 6(4), 435-442.

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K., 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. The Journal of biological chemistry 277(17), 15028-15034.

Bettenworth, D., Nowacki, T.M., Friedrich, A., Becker, K., Wessling, J., Heidemann, J., 2013. Crohn's disease complicated by intestinal infection with methicillin-resistant Staphylococcus aureus. World journal of gastroenterology 19(27), 4418-4421.

Born, G.C.C., 2000. Plantas medicinais de Mata Atlantica (Vale do Ribeira-SP). Tese de doutorado, Universidade de Sao paulo, Sao Paulo.

Cipriani, T.R., Mellinger, C.G., de Souza, L.M., Baggio, C.H., Freitas, C.S., Marques, M.C., Gorin, P.A., Sassaki, G.L., Iacomini, M., 2006. A polysaccharide from a tea (infusion) of Maytenus ilicifolia leaves with anti-ulcer protective effects. Journal of natural products 69(7), 1018-1021.

Dziarski, R., Gupta, D., 2000. Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. Journal of endotoxin research 6(5), 401-405.

Fernandes, P., MacSharry, J., Darby, T., Fanning, A., Shanahan, F., Houston, A., Brint, E., 2016. Differential expression of key regulators of Toll-like receptors in ulcerative colitis and Crohn's disease: a role for Tollip and peroxisome proliferator-activated receptor gamma? Clinical and experimental immunology 183(3), 358-368.

Ferruzza, S., Scarino, M.L., Gambling, L., Natella, F., Sambuy, Y., 2003. Biphasic effect of iron on human intestinal Caco-2 cells: early effect on tight junction permeability with delayed onset of oxidative cytotoxic damage. Cellular and molecular biology 49(1), 89-99.

Gajbhiye, N., Makasana, J., Saha, A., Patel, I., Jat, R., 2016. LC-ESI-MS/MS method for stimulating determination of triterpenoid glycosides and aglycones in *Centell asiatica* L. Chromatographia 79, 727-739.

Hausmann, M., Kiessling, S., Mestermann, S., Webb, G., Spottl, T., Andus, T., Scholmerich, J., Herfarth, H., Ray, K., Falk, W., Rogler, G., 2002. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology 122(7), 1987-2000.

Jorge, R.M., Leite, J.P., Oliveira, A.B., Tagliati, C.A., 2004. Evaluation of antinociceptive, anti-inflammatory and antiulcerogenic activities of Maytenus ilicifolia. J Ethnopharmacol 94(1), 93-100.

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., Kagnoff, M.F., 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. The Journal of clinical investigation 95(1), 55-65.

Kang, S.S., Noh, S.Y., Park, O.J., Yun, C.H., Han, S.H., 2015. Staphylococcus aureus induces IL-8 expression through its lipoproteins in the human intestinal epithelial cell, Caco-2. Cytokine 75(1), 174-180.

Kaser, A., Zeissig, S., Blumberg, R.S., 2010. Inflammatory bowel disease. Annual review of immunology 28, 573-621.

Laparra, J.M., Hernandez-Hernandez, O., Moreno, F.J., Sanz, Y, 2013. Neoglycoconjugates of caseinmacropeptide and galactooligosaccharides modify adhesion of intestinal pathogens and inflammatory response(s) of intestinal (Caco-2) cells. Food Res Int 54, 1096-1102.

Leite, J.P., Rastrelli, L., Romussi, G., Oliveira, A.B., Vilegas, J.H., Vilegas, W., Pizza, C., 2001. Isolation and HPLC quantitative analysis of flavonoid glycosides from Brazilian beverages (Maytenus ilicifolia and M. aquifolium). Journal of agricultural and food chemistry 49(8), 3796-3801.

Li, Z., Xiao, X., Yang, M., 2016. Asiatic Acid Inhibits Lipopolysaccharide-Induced Acute Lung Injury in Mice. Inflammation 39(5), 1642-1648.

Natoli, M., Leoni, B.D., D'Agnano, I., Zucco, F., Felsani, A., 2012. Good Caco-2 cell culture practices. Toxicology in vitro : an international journal published in association with BIBRA 26(8), 1243-1246.

Parveen, I., Wilson, T., Donnison, I.S., Cookson, A.R., Hauck, B., Threadgill, M.D., 2013. Potential sources of high value chemicals from leaves, stems and flowers of Miscanthus sinensis 'Goliath' and Miscanthus sacchariflorus. Phytochemistry 92, 160-167.

Perey, A.C., Weishaar, I.M., McGee, D.W., 2015. The effect of ROCK on TNF-alpha-induced CXCL8 secretion by intestinal epithelial cell lines is mediated through MKK4 and JNK signaling. Cellular immunology 293(2), 80-86.

Szebeni, B., Veres, G., Dezsofi, A., Rusai, K., Vannay, A., Mraz, M., Majorova, E., Arato, A., 2008. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. Clinical and experimental immunology 151(1), 34-41.

Walsh, D., McCarthy, J., O'Driscoll, C., Melgar, S., 2013. Pattern recognition receptors--molecular orchestrators of inflammation in inflammatory bowel disease. Cytokine Growth Factor Rev 24(2), 91-104.

Yang, G.X., Zhang, R.Z., Lou, B., Cheng, K.J., Xiong, J., Hu, J.F., 2014. Chemical constituents from Melastoma dodecandrum and their inhibitory activity on interleukin-8 production in HT-29 cells. Natural product research 28(17), 1383-1387.

Yun, K.J., Kim, J.Y., Kim, J.B., Lee, K.W., Jeong, S.Y., Park, H.J., Jung, H.J., Cho, Y.W., Yun, K., Lee, K.T., 2008. Inhibition of LPS-induced NO and PGE2 production by asiatic acid via NF-kappa B inactivation in RAW 264.7 macrophages: possible involvement of the IKK and MAPK pathways. International immunopharmacology 8(3), 431-441.