



Final report:

***Maytenus ilicifolia* anti-inflammatory screening test**

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The aim of the study was to assess the anti-inflammatory and anti-oxidant properties of *Maytenus ilicifolia*. This was achieved using an *in vitro* human and horse intestinal cell culture model of inflammation.

Introduction

Ulceration of the gastro-intestinal tract affects many mammalian species, including man and the horse. Ulcers may occur in the stomach (gastric), small intestine (duodenal) or hindgut (colonic) in both species (Kuipers *et al.*, 1995; Nadeau and Andrews, 2010). Ulcer pathophysiology is initiated by bacterial contamination of the sensitive gastric mucosa causing inflammation and the production of free radicals. The inflammatory response modulates gastric epithelial cell functioning, leading to increased hydrochloric acid production and damage to the mucosal surface which culminate in ulcer formation (Blaser and Atherton, 2004).

Equine gastric ulcer syndrome (EGUS) is reported to affect up to 90 % of horses (Nadeau and Andrews, 2010). Effective therapies for treatment in horses are namely prescription-only-medicines (POMs), omeprazole or ranitidine (Higgins and Wright, 1995; Reese and Andrews, 2009) that block the production of hydrochloric acid; they do not address the underlying inflammatory response of gastric or duodenal cells. Moreover, these synthetic compounds are expensive, administered only under direction of a veterinary surgeon in the UK, and their effects may not persist beyond medication withdrawal (Reese and Andrews, 2009). Therefore, there is a pressing clinical and commercial need for discovering new, non-toxic compounds with effective anti-inflammatory and anti-ulcerative properties for use in veterinary medicine. Moreover, natural biological compounds rather than

synthetic drugs (Canter et al 2005) confer an advantage when it comes to market acceptability and licensing restrictions for use in a clinical setting. Phytorigins Ltd., produce and supply a commercially successful oral anti-ulcerative supplement for horses that contains the freeze-dried and ground leaves of *Maytenus ilicifolia*. Phytorigins' customers anecdotally report the supplement to be effective in managing and reducing ulcers in horses and its success is enhanced because *Maytenus ilicifolia* is not restricted for use in competition horses under governing body rules (Federation Equestria Internationale; British Horseracing Authority). However, no studies have investigated whether the use of *Maytenus ilicifolia* may be beneficial for preventing or managing human gastric or duodenal ulcers, either alongside existing treatment regimens or alone. Moreover, the importance of nutritional supplements that are non-toxic, natural compounds ('functional foods') as alternatives to synthetic drugs, are being increasingly recognised for their potential in managing human medical conditions (Lobo et al., 2010).

Maytenus ilicifolia is a Celastraceae plant native to Brazil, which for many years has been anecdotally reported to have anti-inflammatory and anti-ulcerative properties (Balbach, 1980; Cruz 1982; Born 2000). Scientific studies of *Maytenus ilicifolia* efficacy have been conducted using mouse or rat models and, although few specifically investigate its effects relating to gastro-intestinal ulcers, those that do, report positive results (Jorge et al, 2004; Leit et al., 2010; Cipriani et al., 2009). There are no published data that supports the anti-inflammatory or anti-ulcerative functioning of *Maytenus ilicifolia* specifically in cells of the gastrointestinal tract for humans or horses.

Our laboratory has access to a human intestinal epithelial cell line (Caco-2) that serves as an *in vitro* model of cell physiology and toxicology and its use is already established in pharmacokinetic and pharmacodynamic clinical studies (Natoli et al., 2012). The Caco-2 cells exhibit a measurable inflammatory response when stimulated by bacterial molecules. As such the ability of putative anti-inflammatories to abrogate this reaction may also be assessed using this model. Specially, when challenged with the bacterial antigen lipoteichoic acid (LTA) Caco-2 cells undergo an inflammatory response by up-regulating gene expression and secretion of cytokines and chemokines, including interleukin 1 β (IL-1 β) and IL-8 respectively (Natoli et al., 2012). The effect of putative anti-inflammatory agents can thus be measured by co-culturing stimulated cells with the compound under investigation and determining whether the gene expression and/or secretion of cytokines and chemokine's are abrogated. Therefore, the Caco-2 cell line offers the first opportunity to determine whether *Maytenus ilicifolia* has anti-inflammatory and anti-oxidant effects specifically in the human gastrointestinal tract. Should this preliminary study provide evidence that *Maytenus ilicifolia* can

abrogate inflammation in these cells, further research and investment is justified to elucidate its pharmacokinetic and pharmacodynamic effects in-depth. Ultimately, this work may be an initial step to progress *Maytenus ilicifolia*-based functional food supplements to the human/horse market.

The aim of the study was to determine the anti-inflammatory properties of *Maytenus ilicifolia* using an *in vitro* human intestinal cell culture model of absorbing enterocytes. The primary objective in order to achieve this aim was to determine whether pre-treatment by *Maytenus ilicifolia* of Caco-2 cells abrogated the inflammatory response resulting from LTA challenge. The inflammatory response was assessed by measuring the secretion and gene expression of inflammatory mediators (IL-8 and IL-1 β) by the Caco-2 cells. Other experiments to determine whether *Maytenus ilicifolia* inadvertently caused cell death or affected normal cell functioning were conducted as additional control measures.

Materials and Methods

Maytenus ilicifolia extract preparation

Freeze-dried and ground *Maytenus ilicifolia* leaves, currently used in a commercial equine nutritional supplement, were provided by Phytorigins Ltd. Extract from the dried and ground *Maytenus ilicifolia* leaves was kindly prepared at Bangor University by Dr Vera Thoss. Briefly, 500 g dried leaves were submitted to hexane percolation and after evaporation in a vacuum rotary evaporator at 45 °C, hexane extract was obtained. The extract was then subjected to a second extraction in ethyl acetate and condensed. The hexane and ethyl acetate extract received at our laboratory totalled 10 g (2 % yield).

Caco-2 cell culture

Frozen Caco-2 cells from passage 100 were generously donated by Dr Igea D'Agnano (CNR-IMCB Monterotondo, Rome). A 1 mL vial of frozen cells were thawed in a 90 mm tissue culture dish and maintained in complete medium (DMEM supplemented with 4 mM glutamine, 100 μ /mL penicillin, 100 UI/mL streptomycin, 1 % non-essential amino acids and 10 % heat inactivated foetal calf serum). Cells were maintained at 37 °C in a 90 % air and 10 % CO₂ atmosphere. When cells reached 50 % confluence the medium was removed and cells were washed with 2 mL of trypsin-EDTA (0.25 % trypsin, 10 mM EDTA in phosphate buffered saline). The dishes were then supplemented with 2 mL of fresh trypsin-EDTA solution and left for 4 min in the incubator. The action of trypsin was stopped by addition of 2 mL of complete medium and the cell suspension was transferred to a 15 mL tube and centrifuged for 5 min at 180 x g. After removing the supernatant, the cell pellet was re-suspended in complete medium and the cells seeded at 4.5 x 10³ cells/cm². Medium was changed every 48 hours.

Plating out protocol

For the experiments using cycling cells a total of 0.5×10^5 cells/cm² were seeded in complete medium in a 12 well plate; experiments were commenced 24 hours after seeding in order to avoid the cell line reaching confluence during the time course. For the differentiation experiments, cells were seeded on PET transwell membrane inserts (4.71 cm² area) at a density of 3×10^5 cell/cm² and maintained for 21 days in complete medium; in these conditions cells reach confluence in 3 days and differentiate completely in 21 days. Therefore, experiments commenced at the end of the 21st day of differentiation.

Trans-epithelial electric resistance (TEER)

The integrity of the differentiated monolayer in terms of permeability of the tight junctions was determined by measuring the TEER of cell monolayers using a commercial apparatus (Millicell ERS; Millipore Co., Bedford, MA) employing Ag–AgCl electrodes, according to manufacturer's instructions. The final values were expressed as $\Omega \cdot \text{cm}^2$ on the basis of the following equation: $\text{TEER} = (R - R_b) \cdot A$, where R is the resistance of filter insert with cells, R_b is the resistance of the filter alone and A is the growth area of the filter in cm².

Phenol red apparent permeability (Papp)

Phenol red apparent permeability was measured as previously described (Ferruzza et al., 2003). The trans-epithelial flux was expressed as the apparent permeability coefficient (Papp), which is reported in units of mass per area per unit time according to equation: $\text{Papp} = K \cdot V_t \cdot A^{-1}$ where K is the steady-state rate of change in concentration in the receiver chamber versus time, $K = (C_t/C_0 \cdot t)$ where C_t is the concentration in the receiver compartment after 60 min, C_0 is the initial concentration in the AP chamber, V_t is the volume of the receiver chamber (mL), A is the surface area of the filter membrane (cm²) and t is the time (s).

Experimental design

The effect of *Maytenus ilicifolia* on cells stimulated with LTA (a stimulant of inflammation) or hydrogen peroxide (H₂O₂; a stimulant of oxidation) was assessed by measuring cytokine and chemokine gene expression and secretion. In addition, gene expression of Caspase 3 (a marker of cell apoptosis) and cell staining for KT18 (a caspase cleavage product of Cytokeratin 18) were undertaken to demonstrate that data generated were not confounded by cell death. All experiments were conducted on Caco-2 cells during cycling and differentiated stages.

To investigate whether *Maytenus ilicifolia* possessed anti-inflammatory properties, cells were cultured in medium alone (0 control), or pre-treated with 12.5, 25, 50, 100 or 200 µg/mL *Maytenus ilicifolia* extract. This time point of first cell treatment was designated 0 hours. These cells were incubated for 24 hours after which medium was removed and replaced with fresh medium containing the control or the same concentrations of *Maytenus ilicifolia*, or *Maytenus ilicifolia* in combination with 5 µg/mL LTA for cycling cells or 15 µg/mL for differentiated cells in the apical compartment only. Plates were returned to the incubator for a further 24 hours. Therefore forty eight hours after the first cell treatment, cells and cell supernatants were harvested.

Supernatants were collected and stored at -80 °C until analysed for cytokine or chemokine secretion by ELISA. The remaining cells were lysed in Lysis buffer and stored at -80°C until used for total RNA purification and reverse transcription to cDNA. Each experiment repeated using three separate passages (passage 102, 103 and 104), and within each passage, experiments were conducted in duplicate.

ELISAs

The IL-8 and IL-1β concentration in culture medium was quantified using a DuoSet ELISA kit by R&D and performed according to manufacturer instructions. The optical density was read with an iMark microplate reader (Bio-Rad) at 450 nm and the raw data were analysed by applying a 4 parameter logistic curve (4-PL) with MPM6 software (Bio-Rad). The samples were run in duplicate.

Total RNA preparation and reverse transcription to cDNA

Total RNA was purified using the Total RNA Purification Plus Kit supplied by Norgen and performed accordingly to manufacturer instructions. Isolated RNA was quantified using a NanoDrop ND-1000 and the quality was estimated on the ratio A260/280 and A260/230. A total of 250 ng of total RNA was retro-transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using random primers. The reaction was performed in a volume of 20 µL and the cDNA obtained was diluted 5 times in preparation for qPCR.

qPCR protocol and primer sequences

The cDNA was subjected to real-time PCR analysis using a Bio-Rad CFX-Connect thermal cycler, Maxima SYBR green/ROX Master Mix (Thermo) and the following specific primer pairs: IL-1β (s: TACCTGTCCTGCGTGTTGAA, as: TCTTTGGGTAATTTTTGGGATCT), CASP3 (s:

TGTGAGGCGGTTGTAGAAGA, as: GGGCTCGCTAACTCCTCAC). Experiments were performed in technical duplicates and in biological triplicates. Gene expression data were normalized using the Cq values of the internal controls PPIA and TBP.

Immunofluorescence protocol for KT18 staining.

The mouse monoclonal antibody M30 for the detection of the caspase cleavage product of Cytokeratin 18 was purchased from Roche. The immunofluorescence was performed according to the protocol sheet provided with the antibody. In order to detect the nuclei the cell preparation was also stained with a solution of 4',6-diamidino-2-phenylindole (DAPI, 1 µg/mL) in PBS for 5 minutes. At the end of the nuclei staining the cells were washed three times for 5 minutes each with PBS alone, then briefly rinsed in bi-distilled water and mounted in Prolong Gold anti-fade mounting medium (Invitrogen). The images were acquired using a Leica CTR6000 fluorescence microscope with a 10x dry objective. The settings were maintained constantly among treatments and the focus was adjusted on nuclei (blind acquisition).

Statistical analysis

Data analysis was performed using SPSS version 21.0 and values reported as the arithmetic mean \pm SEM. Analysis of variance (ANOVA) was used to compare cytokine or chemokine production or gene expression fold induction between control and treated cells. A Dunnet's post hoc test was used because there were more than five treatments for each parameter measured. For statistical analysis, IL8 concentrations were normalised by \log_{10} transformation, with one exception (cycling cells, LTA versus 0 control) to achieve a normal distribution and variance homogeneity. Statistical significance was assumed if $P < 0.05$.

Results

Trans-epithelial electric resistance (TEER) and Phenol red apparent permeability (Papp)

Maytenus ilicifolia was not toxic to differentiated cells at all concentrations used in terms of cell to cell integrity or cell membrane permeability (Figure 1).

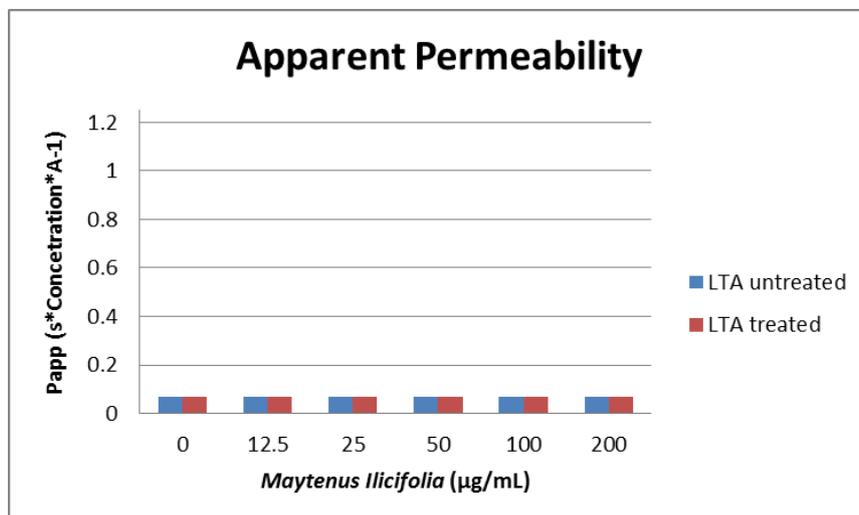
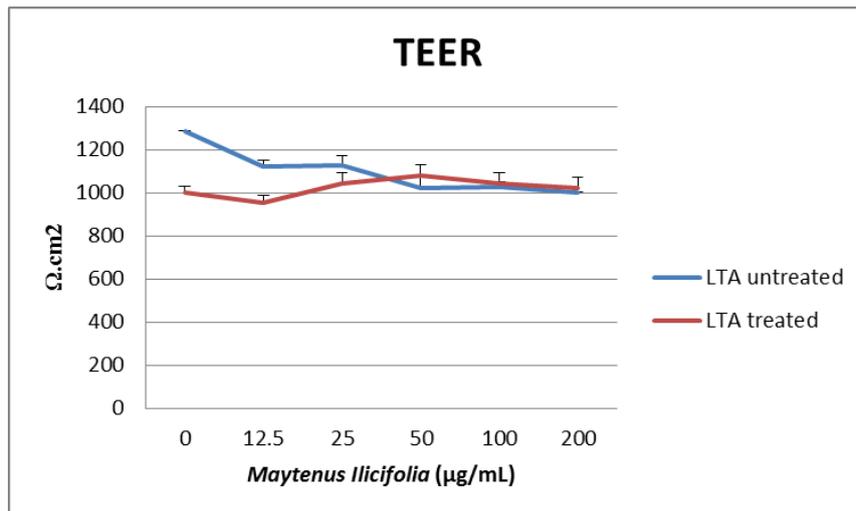


Figure 1: Effect of the pre-treatment with *Maytenus illicifolia* on differentiated Caco-2 cells followed by 24 hour treatment with LTA on the Trans Epithelial Electric Resistance (TEER) and Apparent Permeability (Papp) of the monolayer demonstrated no toxicity at any concentration.

qRT-PCR of Caspase 3 in differentiated Caco-2 cells treated with Maytenus illicifolia from 0 to 200 μg/mL

Differentiated cells: Cells treated with *Maytenus illicifolia* alone or *Maytenus illicifolia* and LTA, did not exhibit increased rates of cell death compared to the untreated (0 control), as determined by gene expression of Caspase 3 (Figure 2).

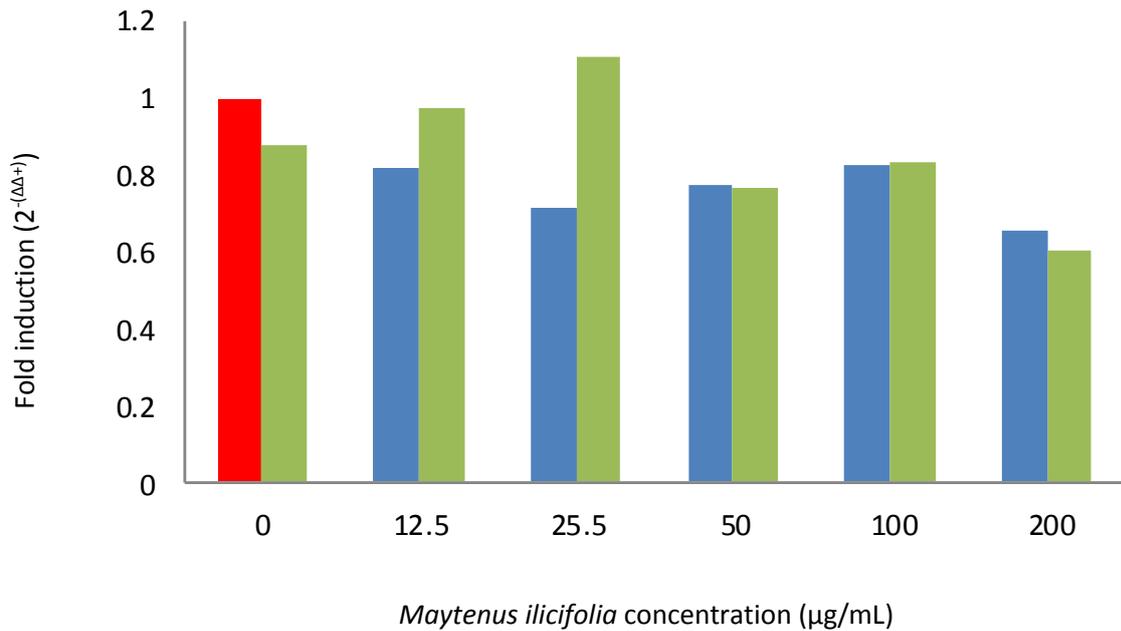


Figure 2: Gene expression of caspase-3 by differentiated Caco-2 cells did not differ between untreated 0 control () or with cultured with increasing concentrations of *Maytenus ilicifolia* alone () or *Maytenus ilicifolia* with () 15 ng/ml A.

Cycling Caco-2 cells treated with Maytenus ilicifolia undergo apoptosis only at the higher concentration
 The immunofluorescence staining with the monoclonal antibody anti Cytokeratin 18 shows an increasing rate of positive cells in the sample treated with 200 µg/mL of *Maytenus ilicifolia* (Figure 3). For this reason the experiments on cycling cells were conducted using concentrations only in the range 0 - 100µg/mL.

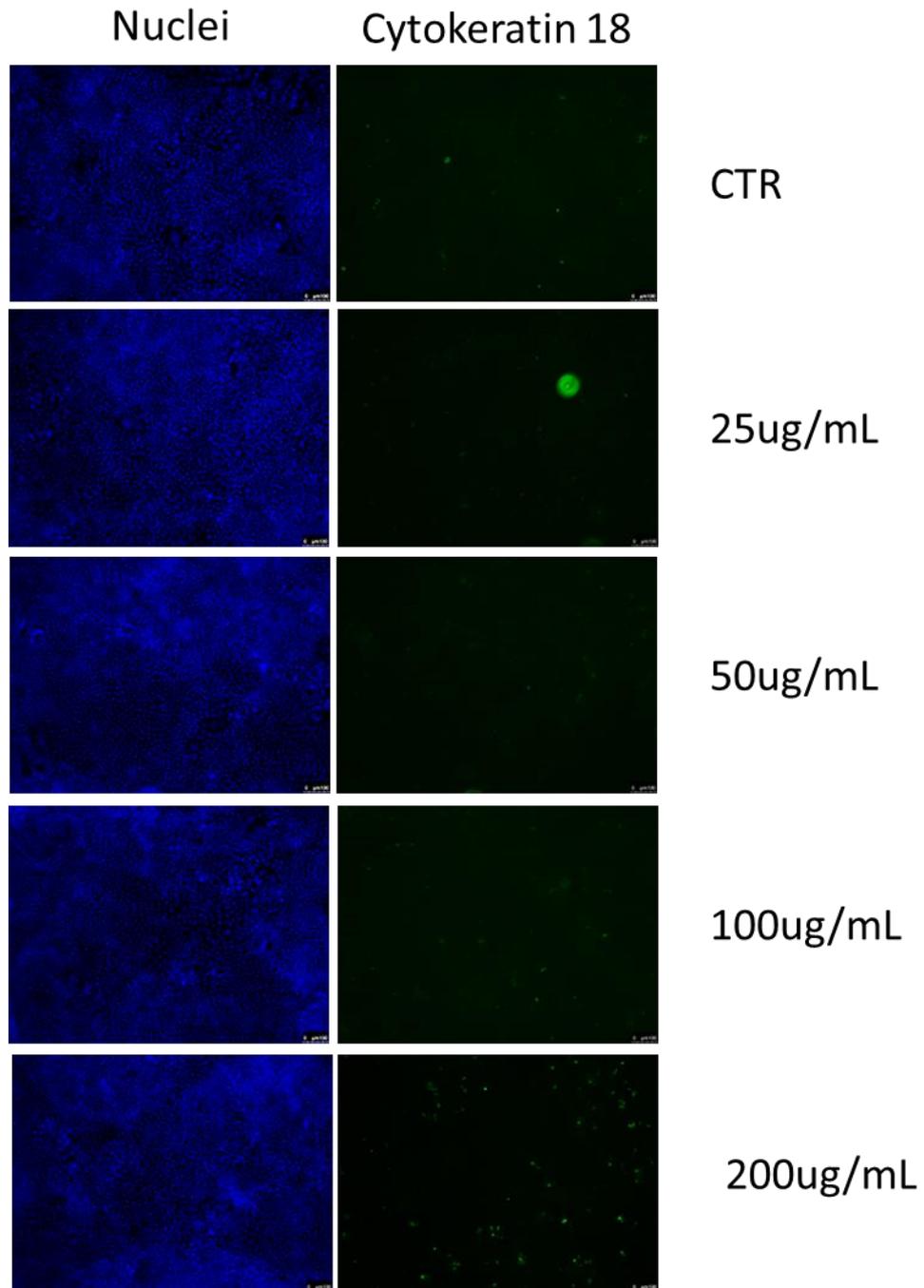


Figure 3: Cycling caco-2 cells stained for cytokeratin-18 show increased uptake only in those treated with 200 $\mu\text{g}/\text{mL}$ *Maytenus ilicifolia*.

Interleukin-1 β gene expression by stimulated Caco-2 is abrogated by Maytenus ilicifolia

Differentiated cells: Gene expression of IL-1 β by Caco-2 cells in the presence of *Maytenus ilicifolia* alone did not increase compared to untreated controls. Caco-2 cell gene expression of IL-1 β increased when stimulated by 15 ng/ml LTA compared to untreated (0 control). However, up-regulation of IL-1 β

gene expression by Caco-2 cells stimulated with LTA was abrogated by *Maytenus ilicifolia* in a dose dependent manner (Figure 4).

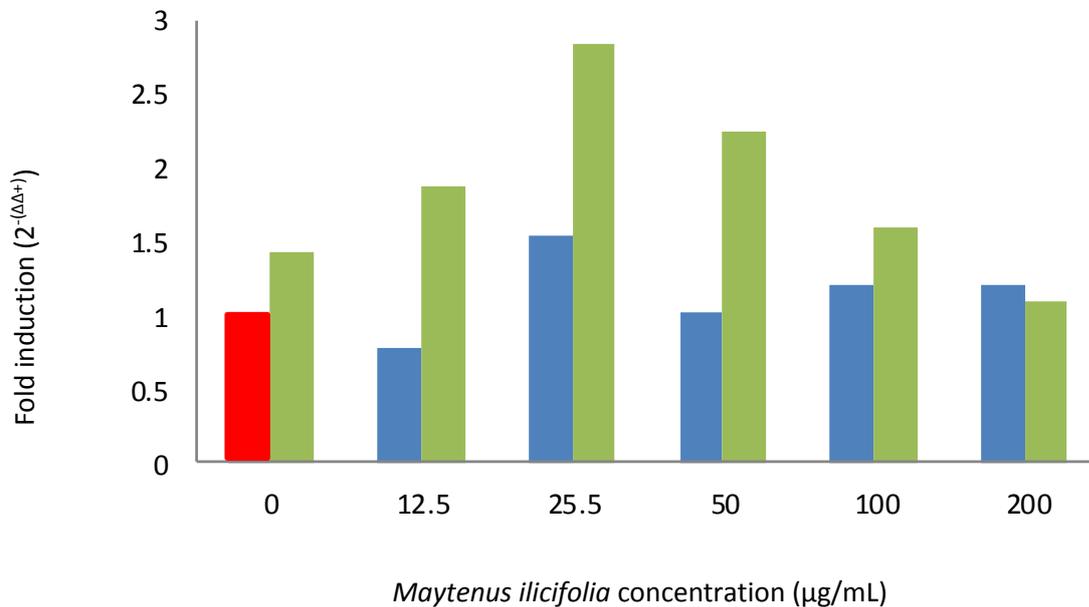


Figure 4: Gene expression of IL-1 β by differentiated Caco-2 cells treated with medium alone (untreated 0 control ■) or cultured with *Maytenus ilicifolia* alone (■) or *Maytenus ilicifolia* in the presence of 15 ng/ml LTA (■).

Interleukin-8 secretion by stimulated Caco-2 when treated with Maytenus ilicifolia

Cycling cells: There were no significant differences for IL-8 secretion between the cells cultured in medium alone (0 control) and those treated with all concentrations of *Maytenus ilicifolia* ($P > 0.05$). Cells treated with LTA and 100 $\mu\text{g/mL}$ *Maytenus ilicifolia* secreted significantly less IL-8 compared with those treated with LTA alone ($P < 0.05$; Figure 3). Cells treated with LTA alone or LTA in combination with 12.5, 25 or 50 $\mu\text{g/mL}$ *Maytenus ilicifolia* secreted significantly increased IL8 concentrations compared to the 0 control ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.01$ respectively; Figure 3). However, there was no significant difference of IL-8 secretion between cells treated with LTA in combination with 100 $\mu\text{g/mL}$ *Maytenus ilicifolia* compared to the 0 control ($P > 0.05$; Figure 5).

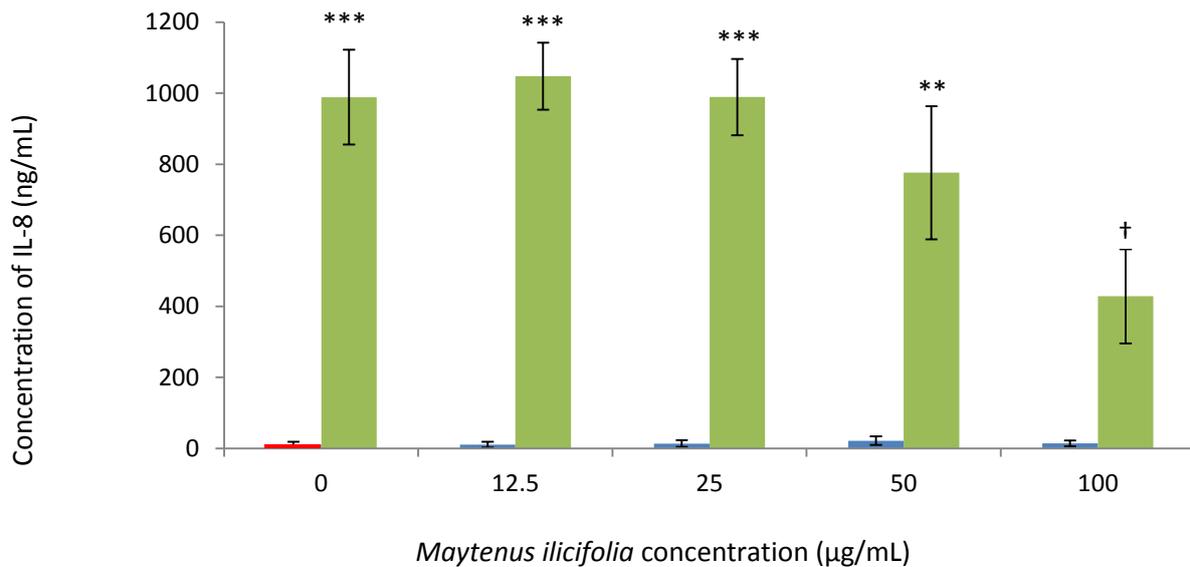


Figure 5: Secretion of IL-8 from cycling CaCo-2 cells treated with medium alone (untreated 0 control ■) or cultured with *Maytenus ilicifolia* alone (■) or *Maytenus ilicifolia* in the presence of 5 ng/ml LTA (■). Values differ significantly from 0 control (medium alone) ** P < 0.01, *** P < 0.001. Values differ significantly from cells treated with LTA but without *Maytenus ilicifolia* †P < 0.05.

Differentiated cells: There were no significant differences of IL-8 secretion between the cells cultured in medium alone (0 control) and those treated with all concentrations of *Maytenus ilicifolia* ($P > 0.05$). There were no significant differences for IL-8 secretion between cells treated with LTA in combination with all *Maytenus ilicifolia* compared to LTA alone ($P < 0.05$). There was a tendency for cells treated with LTA alone to secrete significantly increased IL-8 concentrations compared to the 0 control ($P < 0.08$; Figure 6). Cells treated with LTA in combination with 12.5 or 25 µg/mL *Maytenus ilicifolia* secreted significantly increased IL-8 concentrations compared to the 0 control ($P < 0.05$; Figure 6). However, there was no significant difference of IL-8 secretion between cells treated with LTA in combination with 50, 100 or 200 µg/mL *Maytenus ilicifolia* compared to the 0 control ($P > 0.05$; Figure 6).

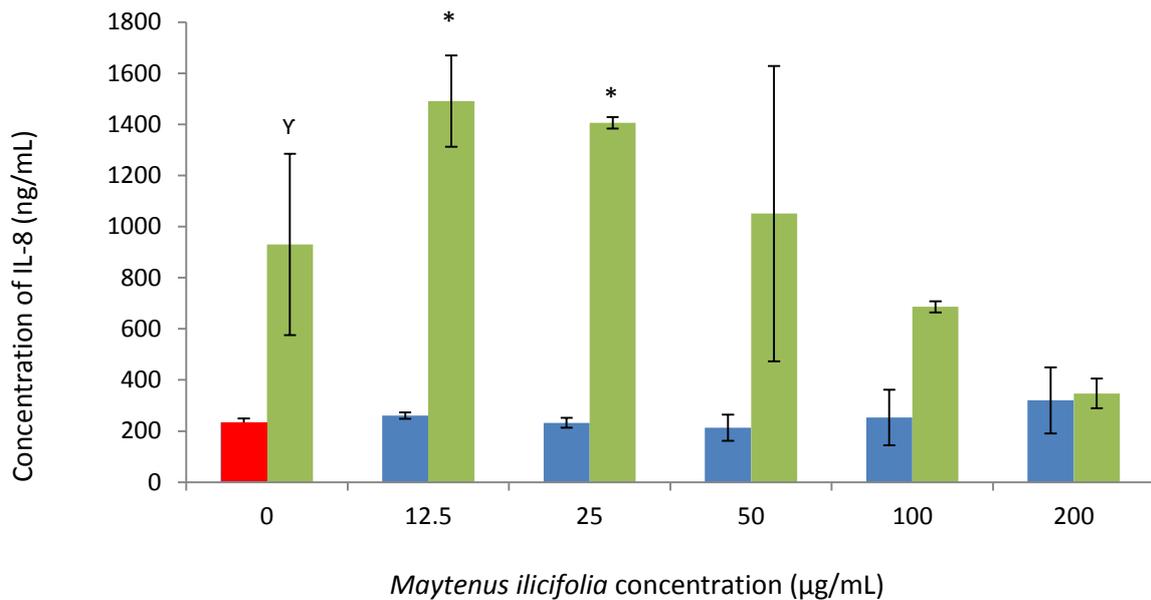


Figure 6: Secretion of IL-8 from differentiated Caco-2 cells treated with medium alone (untreated 0 control) or cultured with *Maytenus ilicifolia* alone () or *Maytenus ilicifolia* in the presence of 15 ng/ml LTA (). Values differ significantly from 0 control (medium alone) * $P < 0.05$. Values had a tendency to differ from 0 control (medium alone) $^Y P < 0.08$.

Discussion

Maytenus ilicifolia did not cause Caco-2 cell death on differentiated cells in the range of concentration from 0 to 200 µg/mL, as determined by gene expression of the cell apoptosis marker, Caspase-3. Furthermore, *Maytenus ilicifolia* did not alter differentiated cell functioning as determined by the permeability properties of the epithelia monolayer as demonstrated by the Apparent Permeability and Trans Epithelia Electric Resistance. Importantly, when Caco-2 cells were induced by bacterial molecules to enter a state of inflammation, *Maytenus ilicifolia* was able to abrogate the response at concentrations of 100 µg/mL or greater. The inflammatory response, and subsequent abrogation of it by *Maytenus ilicifolia*, was measured using markers: gene expression of the inflammatory cytokine IL-1 β , and secretion of the chemokine IL-8, by Caco-2 cells. The study provides preliminary yet promising evidence that *Maytenus ilicifolia* does not accelerate death or inhibit normal functioning of human differentiated intestinal cells and indeed, exhibits anti-inflammatory properties effective at abrogating inflammation in the event of immune challenges to these cells.

To our knowledge, this is the first study to confirm that *Maytenus ilicifolia* does not have a negative effect differentiated cells in terms of causing apoptosis, at least using the well-established, Caco-2 human intestinal cell line. Apoptosis, or programmed cell death, is the process whereby host cells

infected or activated by foreign molecules die without releasing their contents to limit spread of infection or the damaging effects of activating extra-cellular inflammatory processes. Activation of the proteolytic caspase enzyme cascade is an essential intracellular mechanism for activating apoptosis (Benjamin et al., 2000). Therefore, gene expression of members of the caspase family, including Caspase-3, increase during apoptosis. Should *Maytenus ilicifolia* be toxic to Caco-2 cells, apoptosis was hypothesised to take place via the caspase system and it would follow that Caspase-3 gene expression would increase. However, this was not the case and as such, *Maytenus ilicifolia* did not inadvertently cause cell death; an essential property for a compound intended as a potential anti-inflammatory therapy to restore cells to a healthy, fully functioning, non-inflammatory state. Furthermore, these data support subsequent experiments conducted in this study; where *Maytenus ilicifolia* was observed to reduce secretion of inflammatory markers, this was not inadvertently because the cells had died and therefore, unable to secrete these markers from the outset.

Both gene expression and secretion of the equivalent inflammatory markers provided further evidence that *Maytenus ilicifolia* functions as an anti-inflammatory. The cells' inflammatory response in terms of IL-1 β (at the level of gene expression) and IL-8 (at the level of secretion of the protein into culture media), were abrogated by pre-treatment with *Maytenus ilicifolia*. These data broadly agree with that of other scientific studies, where inflammatory effects were measured in terms of paw lesions and oedema in mice or using anti-ulcerative experiments in mice and rats (Jorge et al, 2004; Leit et al., 2010; Cipriani et al., 2009). Moreover, to our knowledge, this is the first study to report potential therapeutic properties of *Maytenus ilicifolia* using human intestinal cells, which is directly and biologically relevant to human duodenal and wider gastro-intestinal ulcers. Furthermore, we used a comprehensive range of relevant inflammatory markers, both at the level of the gene and protein secretion to substantiate the findings.

To further validate the preliminary evidence reported here that *Maytenus ilicifolia* may be useful for anti-ulcerative human supplements, additional studies may be conducted. For example, to establish how *Maytenus ilicifolia* is activated in the human, Caco-2 and liver cells (hepatocytes) could be further studied. Our group has access to the only known human hepatocyte cell line able to undergo steatosis and therefore, have the potential to conduct this work to study the metabolism and physiology of the active compound.

In conclusion, the data presented herein has established for the first-time that that *Maytenus ilicifolia* does not cause cell death or impaired functioning in differentiated human/horse small intestinal cells

in vitro. Furthermore, these data substantiate previous reports of *Maytenus ilicifolia* anti-inflammatory properties, but particularly towards human/horse intestinal cells exhibiting active inflammation. In light of the apoptosis demonstrated by cycling cells at higher concentrations of *Maytenus ilicifolia* application (200 µg/mL) but anti-inflammatory efficacy of 100 µg/mL in both cell types, the data at present suggest dosage rates of 100 µg/mL. Therefore, *Maytenus ilicifolia* is a strong candidate for further study into its anti-inflammatory properties with regard to ulcer therapy with potential for development towards the human dietary supplement market.

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