



Evaluation of the in vitro effect of Boldo and Meadowsweet plant extracts on the expression of antimicrobial peptides and inflammatory markers in canine keratinocytes



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ABSTRACT

Dogs with allergies are prone to skin infections and treatments/preventatives to boost innate immune-defenses are beneficial. The aim of this study was to evaluate the effects of Boldo and Meadowsweet extracts on the expression of β -defensins (cBD), cathelicidin (cCath), and pro-inflammatory cytokines in canine keratinocyte.

This study had two phases. Phase I evaluated mRNA expression of *cBD103* and *cCath*, and secretion of cCath, IL-8 and TNF- α by keratinocytes harvested from healthy (n = 5) and atopic (n = 5) age-matched beagles exposed to Boldo (2% to 0.2%) and Meadowsweet (1% to 0.2%) extracts. Phase II focused on atopic keratinocytes (n = 14) exposed to 0.2% Boldo, 0.2% Meadowsweet, and a mixture of 0.1% of both extracts.

Phase I: *cBD103* mRNA (all concentrations) and TNF- α secretion (2% Boldo) were increased in atopic compared with healthy keratinocytes. In atopic keratinocytes, *cBD103* was increased after exposure to 1.5% and 0.2% Boldo. In healthy keratinocytes, 1% and 0.2% Meadowsweet, and 2% Boldo increased and decreased IL-8 secretion, respectively. In atopic keratinocytes, IL-8 increased after exposure to 1% and 0.4% Meadowsweet extract.

Phase II: *cBD103* mRNA increased after exposure to 0.2% Meadowsweet and to 0.1% mixture. cCath was increased after 0.2% Boldo, but decreased after 0.2% Meadowsweet or the 0.1% mixture. TNF- α secretion was decreased after 0.2% Boldo.

It is concluded that low concentrations of both extracts and their combination may have some effects on cCath and *cBD103* without stimulating an inflammatory response. However, more studies are needed to clarify the effects of these extracts on the local immunity.

1. Introduction

In the past few years a significant and alarming increase in antibacterial resistance has been reported in both people and dogs (Kedzierska et al., 2008; DeBoer and Marsella, 2001). Patients with atopic dermatitis (AD) are affected by recurrent skin infections requiring the continuous use of antibiotics leading to the potential selection for bacterial resistance worldwide (Kedzierska et al., 2008; DeBoer and Marsella, 2001).

Antimicrobial peptides (AMPs) are small, mainly cationic, proteins produced by epithelial and/or immune cells in mammals (Braff et al., 2005). They play an important role in the immune-defense against microorganisms (Jenssen et al., 2006). AMPs physically attach on the

microbes' surface and disrupt it through the formation of pores (Boman et al., 1993). Due to their mechanism of action, AMPs have shown very low bacterial, fungal and viral resistance (Jenssen et al., 2006; Marshall and Arenas, 2003). The most commonly studied AMPs are β -defensins (BDs) and cathelicidin (Cath); they are mainly secreted by epithelial cells. To date, only four BDs and one Cath have been identified in human skin. Their expression has been studied in healthy as well as inflamed skin (e.g. AD and psoriasis) (Schitteck, 2011; Asano et al., 2008; Bellardini et al., 2009; Glaser et al., 2009; Harder et al., 2010). An alteration of their production has been hypothesized to be one of the main reasons of recurrent infections in atopic patients (Lai and Gallo, 2009; Nakatsuji and Gallo, 2012). Similar to people, dogs suffer of naturally occurring AD. Recently a canine model for AD has been

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validated as a reliable model for both human and naturally occurring canine AD (Marsella and Olivry, 2003; Marsella et al., 2006; Marsella and Girolomoni, 2009). AMPs have been studied in this model and they mimic the alterations present in human AD (i.e. increased expression of AMP mRNAs in atopic compared with healthy skin) (Santoro et al., 2011a, 2011b; Santoro et al., 2013). Six BDs (cBD1-like, cBD2-like/122, cBD3-like, cBD102, cBD103, and cBD127) and one cathelicidin (cCath) have been identified in canine epithelia (Leonard et al., 2012; Santoro et al., 2011a; Sang et al., 2005; Sang et al., 2007; Erles and Brownlie, 2010). All the AMPs identified in canine skin have proved antimicrobial activity against the most common skin pathogens (Sang et al., 2005; Sang et al., 2007; Santoro and Maddox, 2014).

Plant extracts have been increasingly used in cosmetics to improve skin conditions and in particular to ameliorate AD in people and dogs (Lee et al., 2006; Schempp et al., 2003; El Zawahry, 1977; Reuter et al., 2010; Schmidt et al., 2010; Marsella et al., 2010; Ferguson et al., 2006; Nagle et al., 2001; Olivry et al., 2010). More recently the antimicrobial and anti-inflammatory properties of plant extract formulations containing Hamamelis or Chinese medical herbs have been studied showing promising effects (Gloor et al., 2002; Higaki et al., 1999; Higaki et al., 1997). Other immunologically active molecules have also been individualized in several plants, such as *Peumus boldus* (aka Boldo) and *Spiraea (Filipendula) ulmaria* (aka Meadowsweet). Boldo is an evergreen tree belonging to the Monimiaceae family native of Chile used for many years to treat gastrointestinal illness (Fernández et al., 2009). Meadowsweet is a perennial herb belonging to the Rosaceae family growing in damp meadows. Meadowsweet is native of Europe and Western Asia (Near East and Middle East). This herb contains high amounts of the polyphenol quercetin that has significant anti-inflammatory and antioxidant effects (Drummond et al., 2013a, 2013b; Mamani-Matsuda et al., 2006), as a ubiquitous flavonoid in the human diet (Peterson and Dwyer, 1988). Although very commonly used in traditional and modern human medicine, very little information is present on their efficacy in animals or people. Both plants have been largely studied in human medicine for their anti-inflammatory (i.e. decreased production of interleukin [IL]-1 β , IL-6, IL-2, and Tumor necrosis factor [TNF]- α) (Lanthers et al., 1991; Churin et al., 2008; Drummond et al., 2013a), and antitoxic/antioxidant/hepatoprotective activity (Lanthers et al., 1991; Shilova et al., 2006; Maksimović et al., 2007; Vengerovsky et al., 2011; Russo et al., 2011; Cebović and Maksimović, 2012). Interestingly a significant antimicrobial activity against *Helicobacter pylori* (Pastene et al., 2014) has also been described. Based on the large amount of information regarding the beneficial effects of Boldo and Meadowsweet plant extract, very recently two products containing either *Peumus boldus* (aka Boldo or wild mint) leaf extract and *Spiraea (Filipendula) ulmaria* (aka Meadowsweet) extracts have been formulated.

In the past few years, with the increased presence of antimicrobial resistance, few studies have been done on the evaluation of alternative ways to stimulate or substitute the production of natural defenses, like AMPs. In 2005, Pernet et al. showed that 75 out of 184 plant extracts were able to stimulate the production of hBD2 and hBD3 in primary keratinocytes harvested from healthy individuals (Pernet et al., 2005). Similarly, 60 out of 75 plant extracts were lacking any in vitro pro-inflammatory stimulation in healthy human keratinocytes (Pernet et al., 2005).

Thus, due to the immunological and cellular protective properties of Boldo and Meadowsweet extracts, the aim of this study was to evaluate the mRNA expression of AMPs (cBD103 and cCath) by quantitative RT-PCR (qRT-PCR) and the production of pro-inflammatory cytokines (TNF- α and IL-8) and cCath by ELISA in the supernatant of primary canine keratinocytes harvested from healthy and atopic beagles before and after exposure to such extracts. This study was composed of two phases focused on the investigation on the in vitro immunological effects of Boldo leaves and Meadowsweet entire plant extracts on primary canine keratinocytes. The first phase of this study analyzed the pro-inflammatory and the AMP stimulating effects of Boldo and

Meadowsweet extracts on canine keratinocytes harvested from healthy and atopic beagles. The second phase was based on the results of the first phase; the same outcomes were investigated but focusing only on atopic keratinocytes using the lowest concentrations tested (0.2%) for Boldo and Meadowsweet extracts as well as a combination of 0.1% of both extracts.

2. Materials and methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.1. Animals

2.1.1. Phase I

Five experimentally-sensitized atopic beagle dogs (2 male, 3 female; age: 7 years) belonging to a colony validated as a model for AD were used (Marsella and Olivry, 2003; Marsella et al., 2006; Marsella and Girolomoni, 2009). Five healthy unrelated beagle dogs (2 male, 3 female; age: 7 years) with no history of skin disease or pruritus were included in this study as age- and breed-matched controls.

2.1.2. Phase II

Fourteen experimentally-sensitized atopic beagle dogs (6 male, 8 female; age: 7 years) belonging to the colony validated as a model for AD including the five atopic beagles used in the Phase I were used (Marsella and Olivry, 2003; Marsella et al., 2006; Marsella and Girolomoni, 2009).

2.2. Housing conditions

All dogs were housed indoors in individual temperature- and humidity-controlled cement runs (22–24 °C; relative humidity of 68–72%). The runs were washed daily using a high-temperature and high-pressure wash (water and bleach). Air filters were changed routinely and stuffed toys, carpets, soft bedding or anything that could trap dust were not allowed in the runs. The dogs were fed a commercial premium maintenance diet (Hill's Science Diet Adult Maintenance kibble formulation; Hill's Pet Nutrition Inc., Topeka, KS, USA).

2.3. Skin sample collection

One to three 8-mm skin biopsy punches from the inguinal area from nonlesional areas were collected following subcutaneous injection of 1 mL of lidocaine (Lidocaine HCl 2%, Hospira Inc., Lake Forest, IL, USA) mixed equally with 8.4% sodium bicarbonate. Skin samples, sterilely collected and placed on ice in Dulbecco's phosphate buffered saline (D-PBS), were brought back to the laboratory and placed in betadine solution for 15 min and then washed multiple times in D-PBS.

2.4. Cell cultures

After washed in PBS the skin samples were cut in strips while submerged in serum-free epidermal keratinocyte growth medium (CnT-09[®], CellnTec, Bern, Switzerland). The skin sections were placed in 1.25 U/mL of dispase (Dispase I, Sigma-Aldrich, St. Louis, MO, USA) solution at 4 °C overnight in sterile tubes to detach the epidermis from the dermis, which was teased off and rinsed in D-PBS. The epidermal tissue was then transferred into another Petri dish and laid on a drop of TrypLE™ Select (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C then rubbed on the dish to dissociate basal keratinocytes in added CnT-09[®] medium with fetal bovine serum (FBS). The cell suspension was collected and filtered through a 40 μ m sterile mesh (Fisher Scientific, Pittsburg, PA, USA) then centrifuged at 1100 rpm for 5 min at 25 °C. The cell pellet was then resuspended in 0.5 mL of CnT-09[®] medium and counted. Cells were resuspended in CnT-09[®] enriched with fetal bovine

serum and 8 ng/mL of cholera toxin (Sigma-Aldrich, St. Louis, MO, USA) to 1×10^4 /mL and 500 μ L were placed in 48 well plates precoated with coating matrix (Invitrogen, Carlsbad, CA, USA), then incubated at 37 °C, with 5% CO₂. Cell outgrowth was monitored daily and the medium changed every third day.

Keratinocyte cultures were monitored until they were close to confluent (~80%) and then starved for 24 h using CnT-09® without supplements or cholera toxin. For Phase I the supernatant was removed and the keratinocytes were exposed for 24 h to several concentrations of Boldo (2%, 1.5%, 1%, 0.4%, and 0.2%) or Meadowsweet (1%, 0.4%, and 0.2%) extracts (Virbac, Carros, France). Canine recombinant IL-1 (10 ng/mL) (R & D systems, Minneapolis, MN, USA) and CnT-09® without supplements or cholera toxin were used as positive and negative controls, respectively. Since the Meadowsweet extract was diluted in 50/50 water/butylene-glycol solution, an additional negative control using diluted 50/50 water/butylene-glycol solution in CnT-09® without supplements or cholera toxin was used. After testing several Boldo and Meadowsweet concentrations in Phase I, the lowest concentration of both extracts (0.2%) and a mixture containing 0.1% of both extracts were used for Phase II. The goal of the Phase II was to test the effects of the lowest concentrations and a combination of 0.1% of both extracts on atopic keratinocytes only, since an altered expression/secretion of AMPs has been detected in atopic and not healthy dogs. Thus for Phase II keratinocytes were exposed for 24 h to 0.2% Boldo extract, 0.2% Meadowsweet extract, or a mixture of 0.1% of both extracts. Positive and negative controls were similar to Phase I.

2.5. Plant extracts preparation

Peumus boldus leaves, at a concentration of 5–10%, were suspended in water, filtered, and mixed with butylene glycol, pentylene glycol and xanthan gum and further diluted, in water, to the above mentioned concentrations (e.g. 0.1% contains about 0.005–0.01% of *Peumus boldus* leaves extract). The meadowsweet extract was obtained from *Spiraea ulmaria*; the entire plant, at a concentration of about 2%, was suspended in butylene glycol (50/50), then the soluble phase was separated and the active fraction purified, concentrated, and filtered and further diluted to the above mentioned concentrations (e.g. 0.1% contains about 0.002% of *Spiraea ulmaria* extract).

2.6. Quantitative RT-PCR (cBD103 and cCath)

Total RNA was extracted using the PerfectPure RNA cell culture kit from 5 PRIME (Gaithersburg, MD) according to protocol. Briefly after cell supernatant was removed 400 μ L of lysis buffer from the kit was added to the well, agitated by pipet, then transferred to an RNase free tube. An additional 400 μ L was added to collect residual product and also transferred in the same tube. Steps, including a DNase treatment, were then followed according to manufacturer protocol. Total RNA concentrations were assessed at 260 nm using UV NanoDrop1000® spectrophotometry (Thermo Scientific, Wilmington, DE, USA). Total RNA (0.5–1 μ g) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Sense and antisense primers for each AMP (Table 1) were generated using Primer Designer software

Table 1
Primers used.

Canine gene	Primer sequence	Amplicon length
cBD103	Forward: ACCTTGCCATCGAGTCTCAG Reverse: GGAACAGGCATCAAGAACAG	98
cCath	Forward: CACTGTTGCTACTGCTGCTG Reverse: GTTGAAGCCATTACAGCAC	98
RPLO	Forward: TTGTGGCTGCTGCTCCTGTG Reverse: ATCCTCGTCCGATTCTCCG	107

(Scientific and Educational Software, Inc.) as previously reported (Santoro et al., 2013). The relative mRNA expression levels were quantified using SYBR® Green (Qiagen, Valencia, CA, USA) and ABI (Applied Biosystems Inc., Foster City, CA, USA) quantitative RT-PCR methodology. All samples were tested in triplicate 25 μ L reactions in an ABI 7500 Real Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). PCR amplifications were carried out as followed: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s; 60 °C for 60 s. Amplifications were followed by dissociation (melting) curves to ensure specificity of the primers. The results were analyzed using the comparative C_T (cycle threshold) method, and the relative mRNA expression of each AMP was compared using the $\Delta\Delta C_T$ method ($2^{-[\Delta C_T \text{ experimental sample} - \Delta C_T \text{ control sample}]}$) where ΔC_T is the difference between the target gene and the normalizer gene expression. All samples were normalized against the canine ribosomal protein L15 (RPLO) as previously reported (Santoro et al., 2011a, 2011b).

2.7. Enzyme-linked immunosorbent assay (ELISA) (TNF- α and IL-8)

Sandwich ELISAs were performed according to manufacturer's protocol using Quantikine canine IL-8 and TNF- α kits (R & D Systems, Minneapolis, MN). Cell culture supernatant was tested undiluted for TNF- α , but diluted 1:10 in RPMI media for IL-8 evaluation. The assays were performed in duplicates.

2.8. Relative competitive inhibition ELISA (ciELISA) (cCath)

The amount of cCath secreted by the keratinocyte cultures was quantified using a ciELISA as previously described (Santoro et al., 2013). Briefly, 96-well flat-bottom microtiter plates (Immunolon II® HB, Fischer, Pittsburgh, PA, USA) were coated with 50 μ L/well of 10 ng/mL synthetic cCath in coating PBS (BioRad, Hercules, CA, USA) (pH 7.4) and left overnight at 4 °C. The next day, the plates were blocked with 100 μ L/well of 10% FBS (Midwest Scientific, St. Louis, MO, USA) in PBS for 2 h at room temperature then the blocking solution was discharged. The ciELISA was formed by four sets of wells: 1) Three wells filled with 50 μ L of specific anti-cCath polyclonal serum at 1:16,000 dilution (Santoro et al., 2011a, 2011b) used as positive control to determine the maximum optical density (OD). 2) Three sets of duplicate wells filled with 50 μ L of 10% FBS and 0.05% Tween-20 in PBS (PBS-T) only or 50 μ L of secondary antibody only or 50 μ L of cCath specific pre-immune serum used as negative control to determine the minimum OD. 3) A series of triplicate wells receiving a mixture of specific anti-cCath polyclonal serum (Santoro et al., 2011a, 2011b) dilution and ten-fold serial dilutions (from 100 ng/mL to 0.01 ng/mL) of the appropriate synthetic peptide, used to generate the inhibition standard curves. 4) Three wells receiving 50 μ L of mixtures containing 25 μ L of supernatant and 25 μ L of specific anti-cCath polyclonal serum (Santoro et al., 2011a, 2011b) dilution.

The sera dilutions were chosen among those that gave OD values on the linear portion of the curve obtained from a two-fold serial titration of each serum on plates coated with the appropriate peptide. The supernatant served as a competitive inhibitor for the binding of the antibodies to the synthetic peptides coating the plates. The plates were incubated for 90 min at room temperature, washed five times using the blocking solution and 50 μ L of a secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG at 1:4000 dilution) (BioRad, Hercules, CA, USA) was added and incubated in the dark for 1 h at room temperature. The plates were then washed seven times with blocking solution and 100 μ L/well of ABTS (Kirkegaard and Perry Laboratories, Fischer, Pittsburgh, PA, USA), a colorimetric substrate, was added. The developed plates were read with an automated MR 500 ELISA reader (Dynatech, Chantilly, VA, USA). The percentage of inhibition obtained from each concentration of peptide was calculated using the average of the absorbance values of each triplicate wells and the average of the absorbance values of the positive control wells. To

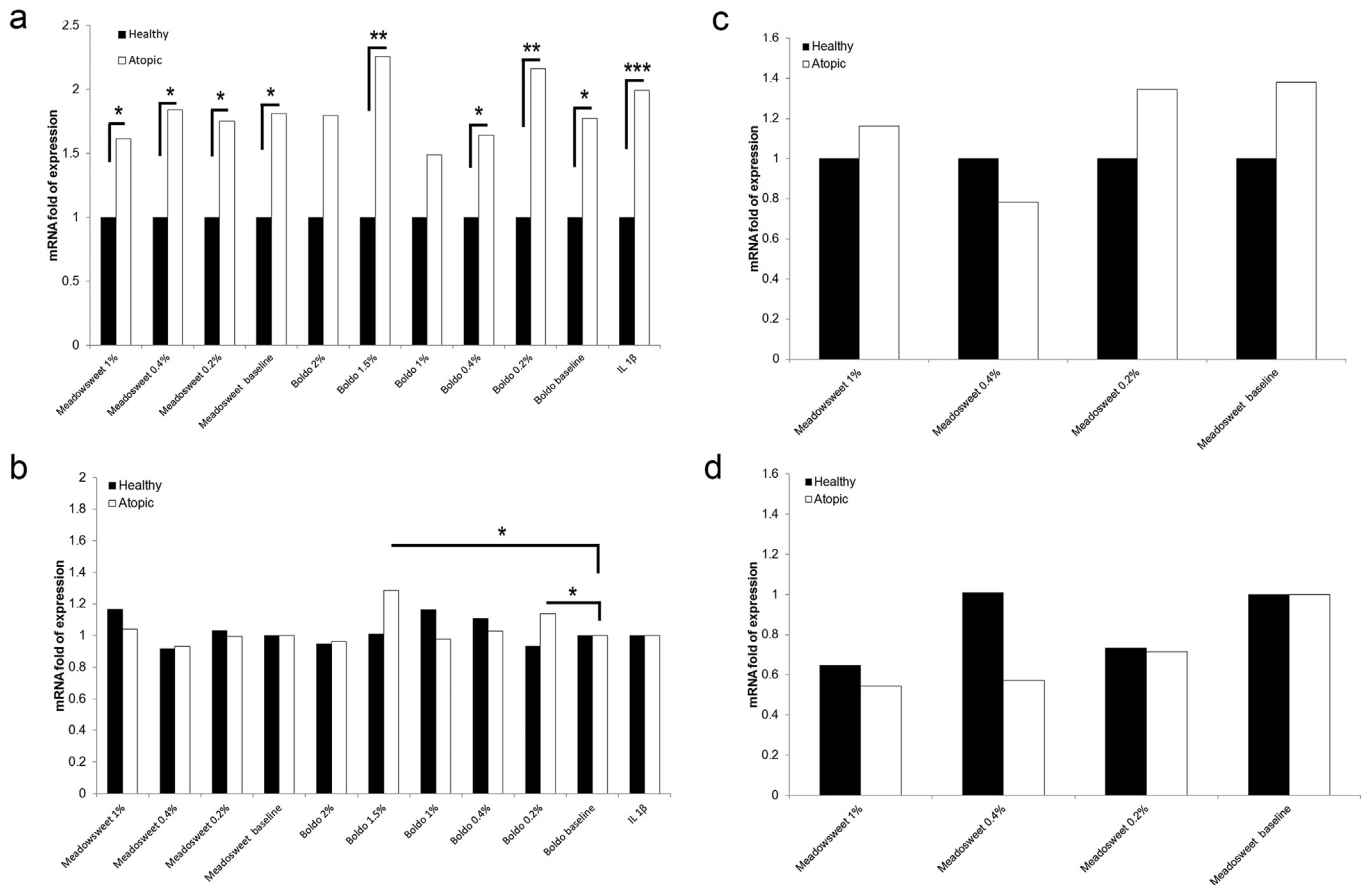


Fig. 1. Phase I - relative mRNA expression for canine β -defensin 103 (a and b) and canine cathelicidin (c and d) in healthy and atopic keratinocytes. a, c) comparison between healthy and atopic keratinocytes; b, d) comparison between before and after stimulation with plant extracts in both groups. Groups were compared using unpaired *t*-test and repeated measurements ANOVA with Dunnett's multiple comparison test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

calculate the relative amount of peptide in the 'unknown' wells, the increasing percentage inhibition values were plotted versus the corresponding log concentration of the synthetic peptide used to generate the standard curves. The relative amount of AMPs in the cell extracts and in the supernatant was expressed as the ng/mL of its synthetic peptide giving the same percentage of inhibition. The *cBD103* protein levels were not quantified due to a lack of antibodies suitable for *ciELISA*-based quantification.

2.9. Statistical analysis

Mean values and 95% confidence intervals were calculated for all results. The Kolmogorov–Smirnov test of normality was used ($\alpha = 0.05$). Differences between ΔC_T (C_T AMP - C_T RPL0) of each AMP and between ODs of IL-8 and TNF- α were compared using Student's unpaired *t*-test (group effect - Phase I). To evaluate the effect of each concentration of Boldo and Meadowsweet extracts on the baseline expression of AMPs and pro-inflammatory cytokine production, a repeated measurement ANOVA was used. The Dunnett's multiple comparison test was used as post-hoc analysis (treatment effect - Phase I). In Phase II, the Student's paired *t*-test was used to compare the effects of each product concentration (Boldo 0.2%, Meadowsweet 0.2%, and mixed of 0.1% Boldo and 0.1% Meadowsweet extracts) on the baseline levels of AMPs and cytokines. Based on preliminary data and expecting higher AMP levels for atopic dogs and no changes in pro-inflammatory cytokines, we used a one-tailed test, and *p*-values of ≤ 0.05 were considered significant. Statistical analysis was done using GraphPad Prism6 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Phase I

In Phase I the *cBD103* mRNA expression was determined before and after exposure to the concentrations of both extracts (Boldo and Meadowsweet). Based on a previous human study (Lenaers et al., 2007), only Meadowsweet extracts' effects were considered for *cCath* mRNA expression. When the mRNA expressions of *cBD103* and *cCath* in atopic keratinocytes were compared with their expression in healthy keratinocytes, a significant difference in *cBD103* (Fig. 1a), but not *cCath* (Fig. 1c), was seen. In particular a significant increase in *cBD103* mRNA was present in atopic compared to healthy keratinocytes at baseline (1.8 times; $p = 0.02$) and after exposure to all concentrations tested of either extracts (range: 1.6–2.2 times), except for 1% and 2% Boldo extracts (Fig. 1a). In addition, *cBD103* mRNA expression was also significantly increased in atopic keratinocytes after exposure to 1.5% (1.3 times; $p = 0.024$) and 0.2% (1.1 times; $p = 0.019$) Boldo extracts when compared to baseline (Fig. 1b). None of the concentrations tested had any significant effect on healthy keratinocytes. No significant differences, after exposure to any of the concentrations tested for Meadowsweet extracts, were detected for *cCath* mRNA or *cCath* secretion in either healthy or atopic keratinocytes (Figs. 1d and 2c).

When the secretion levels of TNF- α and IL-8 in atopic keratinocytes were compared with their secretion levels in healthy keratinocytes, a significant increased production was only detected for TNF- α after stimulation with high concentration (2%) of Boldo extract ($p = 0.0056$) (Fig. 2a). In addition, when the secretion of TNF- α and IL-8 after exposure were compared with their baselines, significant

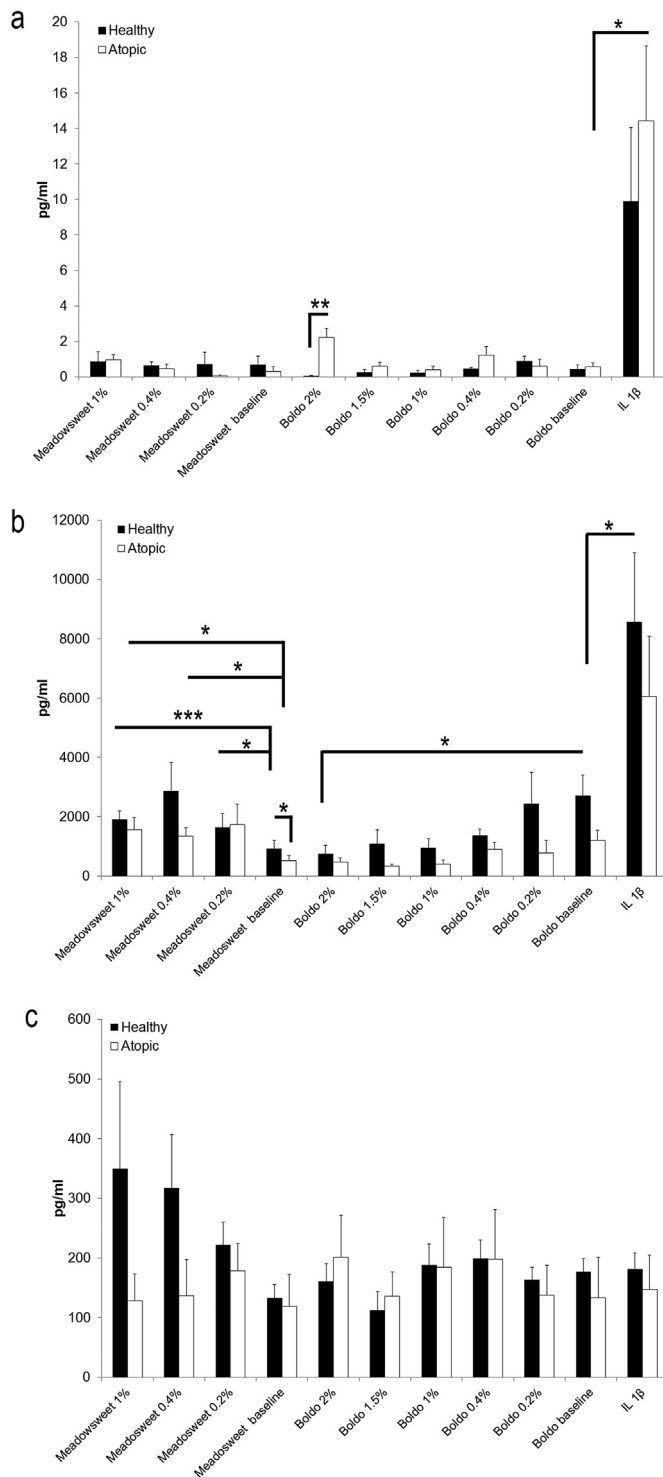


Fig. 2. Phase I - protein levels for canine tumor necrosis factor (TNF)- α (a), interleukin (IL)-8 (b), and canine cathelicidin (c) in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hour stimulation using plant extracts. Groups were compared using unpaired *t*-test and repeated measurements ANOVA with Dunnett's multiple comparison test. **p* \leq 0.05; ***p* \leq 0.01; ****p* \leq 0.001.

differences were detected only for IL-8 (Fig. 2b). In particular, an increase in IL-8 release was seen in healthy keratinocytes after exposure to 1% and 0.2% Meadowsweet extract (*p* = 0.0003 and *p* = 0.031, respectively) while a decrease in IL-8 secretion was seen after exposure to 2% Boldo extract (*p* = 0.025) (Fig. 2b). In atopic keratinocytes, IL-8 secretion significantly increased after exposure to 1% and 0.4%

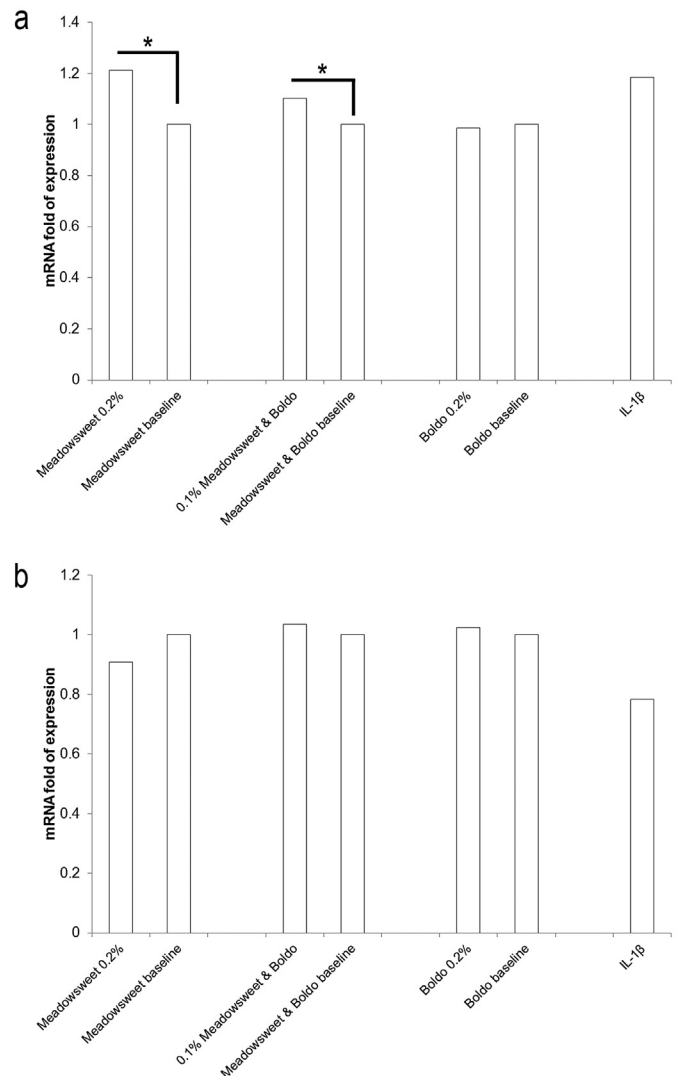


Fig. 3. Phase II - relative mRNA expression for canine β -defensin 103 (a) and canine cathelicidin (b) in atopic keratinocytes before and after stimulation with plant extracts. Groups were compared using paired *t*-test. **p* \leq 0.05.

Meadowsweet extract (*p* = 0.025 and *p* = 0.015, respectively) (Fig. 2b).

3.2. Phase II

Phase II following the Phase I was focused only on atopic keratinocytes. Since an increase in *cBD103* mRNA – associated with no pro-inflammatory effects - were detectable in atopic keratinocytes at lower concentration tested, we analyzed the effects of such concentrations (0.2%) of both extracts as well as a combination of 0.1% of both extracts on the atopic cells only. In Phase II both the *cBD103* and *cCath* mRNA expression were determined before and after exposure to the concentrations of both extracts (Boldo and Meadowsweet) (Fig. 3). When the expressions of *cBD103* and *cCath* mRNA after exposure to 0.2% Meadowsweet, 0.2% Boldo, and the 0.1% combination of both extracts were compared with their expression at baseline, a significant difference in *cBD103* (Fig. 3a), but not *cCath* (Fig. 3b), mRNA was seen. In particular, a significant increase in *cBD103* was seen after exposure to 0.2% Meadowsweet extract (1.2 times; *p* = 0.025) and the 0.1% combination of both extracts (1.1 times; *p* = 0.028) (Fig. 3a). When the *cCath* secretion levels in the supernatants were evaluated, a significant decrease in *cCath* was seen after exposure to 0.2% Meadowsweet extract (*p* = 0.0026) and the 0.1% combination of both extracts

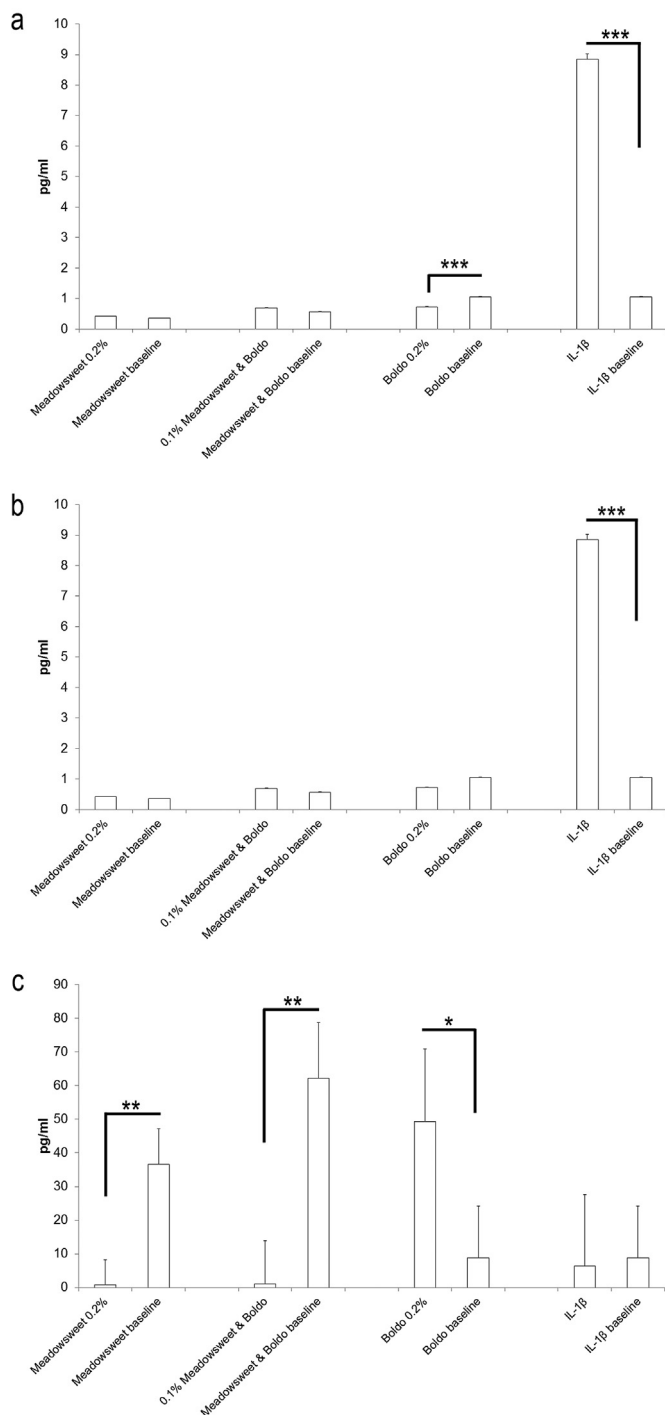


Fig. 4. Phase II - protein levels for canine tumor necrosis factor (TNF)- α (a), interleukin (IL)-8 (b), and canine cathelicidin (c) in the supernatant from keratinocytes harvested from atopic beagles collected before (control) and after 24 hour stimulation using plant extracts. Groups were compared using paired *t*-test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

($p = 0.0017$) (Fig. 4c). On the contrary, 0.2% Boldo extract significantly increased the secretion of cCath in the supernatant ($p = 0.026$) (Fig. 4c).

When the secretion levels of TNF- α and IL-8 were compared with their secretion levels at baseline, no significant differences were seen after exposure to any product, except for 0.2% Boldo extract (Fig. 4a,b). Indeed, a significant decreased secretion of TNF- α was evident after stimulation with 0.2% Boldo extract ($p = 0.00015$) compared to baseline (Fig. 4a).

4. Discussion

The results of the Phase I of this study are in line with previous studies (Lanhers et al., 1991; Churin et al., 2008; Drummond et al., 2013a) showing no inflammatory stimulation by plant extracts along with the possibility to increase the production of AMPs by healthy keratinocyte cell cultures (Pernet et al., 2005). In addition, these results showed for the first time that no pro-inflammatory effects were seen with any concentrations of Boldo extract (alone or in association with meadowsweet extract) in atopic canine keratinocytes (Phases I and II).

The results of the first phase of this study were somewhat confirmed by the second phase of this study focused only on the effects of the lowest concentrations of Boldo (0.2%) or Meadowsweet (0.2%) or a mixture of 0.1% of both extracts on atopic canine keratinocytes. This phase showed no pro-inflammatory effects of any extract or combination of them on atopic keratinocytes. However, a significant decrease in TNF- α was present in atopic keratinocytes after exposure to 0.2% Boldo extract. The lack of anti-inflammatory effects by Meadowsweet extracts on healthy and atopic keratinocytes could be due to a lower concentration (dilution of phenols in the final extract concentrations) used in this study compared with previous studies in which the purified phenols were used (Churin et al., 2008; Drummond et al., 2013a). In addition, some discrepancies regarding the stimulation of TNF- α and Boldo extract, between the two phases of this study are present. In particular, although a lack of effect of 0.2% Boldo extract on TNF- α in atopic keratinocytes, was seen in the Phase I, a significant decrease of this cytokine was seen in Phase II. This difference could be due to the increased number of atopic dogs used in the second phase of this study.

Furthermore, to confirm the absence of pro-inflammatory effects of both plant extracts, this study analyzed the immunological properties, as stimulators of the innate immune-defenses, of Boldo and Meadowsweet extracts on the mRNA expression of endogenous canine AMPs. In the first phase of this study an increased expression of *cBD103*, but not *cCath* was present in atopic keratinocytes only after the exposure to 0.2% and 1.5% Boldo extracts with a dose-independent manner. The positive effect of only these two concentrations and no other ones could be due to a high variability of the data and a low number of dogs enrolled in this study. In addition, a higher expression of *cBD103* was present in atopic keratinocytes when compared to healthy keratinocytes. This difference was statistically significant before and after stimulation with any concentrations of either product except for 1% and 2% Boldo extracts for which only a trend to significance was detected ($p = 0.088$ and 0.066 , respectively). These results are in agreement with previous studies in which a higher expression and production of AMPs was detected in atopic dogs compared with healthy dogs (Santoro et al., 2011a, 2011b, 2013, 2015). In addition, these results are in line with Pernet et al. (2005) showing the possibility for plant extracts to be able to stimulate the production of AMPs in people and dogs.

Similarly to the Phase I, also in the second phase of this study a statistically significant increase in mRNA expression was seen for *cBD103* but not for *cCath*. In particular, an increase in *cBD103* was seen after stimulation with 0.2% Meadowsweet extract or a combination of 0.1% of both extracts. This result was not evident in the Phase I probably due to the low number of dogs used in that phase. However, the 0.2% Boldo extract was able to positively stimulate the production of cCath in atopic keratinocytes while 0.2% Meadowsweet and a combination of 0.1% of both extracts did inhibit cCath secretion. These results confirm the possibility to increase the production of AMPs not only in healthy canine keratinocytes, as demonstrated in people (Pernet et al., 2005), but also in atopic canine keratinocytes. More interesting, these results add to the intrinsic antibacterial property of the Boldo plant extracts already demonstrated (Pastene et al., 2014) of actively stimulating the expression of AMPs in atopic keratinocytes. This latter effect is extremely important since AMPs are very rarely associated with antimicrobial resistance (Jenssen et al.,

2006; Marshall and Arenas, 2003) and atopic patients are commonly affected by bacterial infections, although the association between AMPs secretion and occurrence of skin infections has not been completely elucidated yet.

5. Conclusions

In conclusion, this study showed that Boldo and Meadowsweet plant extracts have no pro-inflammatory properties, based on the lack of stimulation of TNF- α and IL-8, when used in low concentrations in atopic keratinocytes. In addition, these compounds have the potential to modulate the expression of AMPs in canine atopic keratinocytes. These results make 0.2% Boldo extract, 0.2% Meadowsweet extract, and a combination of 0.1% of both extracts a potential perspective for the control of bacterial overgrowth in atopic dogs. Thus the opportunity to increase intrinsic immune-defenses in allergic individuals, and not only in healthy ones, may open new possible uses of plants extracts in the management of cutaneous skin infections. An in vivo double-blinded, randomized, and placebo-controlled clinical trial to evaluate the clinical potentials of these compounded is warranted.

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Conflict of Interest

C. Navarro received reimbursements, fees, funding or salary from: Virbac, H. Gatto received reimbursements, fees, funding or salary from: Virbac, R.

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