An Ayurvedic herbal extract inhibits oral epithelial cell IL-8 responses to host and bacterial agonists

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Abstract

Background: Natural products constitute a promising class of therapeutics for the treatment of gingivitis and periodontitis as well as the maintenance of oral health. However, the limited understanding behind their potential mechanisms and modes of action have hampered their incorporation into popular western therapeutics. This in vitro study characterizes an Ayurvedic herbal extract mixture, which has been clinically shown to promote gingival health and homeostasis.

Methods: Telomerase immortalized gingival keratinocytes (TIGK) were infected with either Fusobacterium nucleatum cell wall, live F. nucleatum, IL-1β or TNF-α for 4 hours with and without the herbal extract. The immunomodulatory effects of the extract on host IL-8 production was measured by ELISA.

Results: It was found that the Ayurvedic herbal extract mixture inhibited gingival epithelial cell IL-8 expression in response to both bacterial and host cytokine agonists. The herbal extract inhibited IL-8 stimulated by F. nucleatum cell wall, live F. nucleatum, IL-1β, and TNF-α in a dose-dependent manner that was not a result of host cell death. Furthermore, the extract showed significantly different ID50 doses demonstrating the differential ability to modulate both stimulated and basal IL-8 levels.

Conclusions: In vitro investigation of this herbal extract mixture revealed that it has the ability to modulate gingival epithelial cell IL-8 expression in response to stimulation by bacterial components and host pro-inflammatory signals. This data demonstrates that the reduction in the gingival epithelial cell IL-8 response may in part be responsible for the previously reported ability of the Ayurvedic herbal extract mixture to reduce gingivitis in two separate human clinical studies.

Keywords: Herbal, Interleukin-8, Periodontitis, Oral health, Fusobacterium nucleatum, Inflammation

One-sentence summary

An ayurvedic herbal extract modulates epithelial cell IL-8 expression, a key host defense component in oral health and disease.
interventions and oral maintenance programs to address oral health. Naturopathic medicine potentially represents one area where new oral health regimes may prove to be beneficial. Numerous studies have identified plant extracts that possess potent antibacterial, antifungal, and anti-inflammatory qualities, which inhibit key inflammatory mediators and have been in use for centuries to maintain oral hygiene [5–11].

For example, in Sri Lanka, a time tested and proprietary Ayurvedic recipe of blended plant extracts has been incorporated into a toothpaste (Sudantha5). The plants used in this product are: heartwood of cutch tree (Acacia chundra Willd.), malabar nut leaf (Adhatoda vasica Nees.), Spanish cherry bark (Mimusops elengi L.), black pepper (Piper nigrum L.), pongam oil tree root (Pongamia pinnata (L.) Pierre), Aleppo oak galls (Quercus infectoria Olivier), clove (Syzygium aromaticum L.), myrobalan fruit (Terminalia chebula Retz.), and ginger (Zingiber officinale Roscoe) and have been used traditionally in Ayurveda for oral care [12–18]. This product has been examined in two separate randomized double-blind placebo-controlled clinical trials. In one human trial, the effects of this herbal extract on oral hygiene and gingival health showed significant reduction of gingival bleeding, dental plaque formation, and salivary anaerobic bacterial counts as early as 4 weeks of its use compared to the placebo group [19]. Moreover a follow-up clinical trial investigating these therapeutic benefits for patients with gingivitis confirmed these results, reporting a reduction in gingival bleeding, plaque score, total salivary anaerobic bacterial counts, and probing pocket depth [9]. Altogether, these randomized clinical studies provide robust evidence of the effective antiplaque and anti-gingivitis effects of this herbal extract for both the maintenance of health and treatment of disease.

Interleukin-8 (IL-8) is a key inflammatory mediator involved in chemotaxis [20] and activation [21] of immune cells, such as neutrophils, as well as promotion of tissue remodeling and angiogenesis [22]. In humans, gingival keratinocytes have been shown to express IL-8 in response to oral bacteria [23], including the “bridging” organism Fusobacterium nucleatum [24, 25], and pro-inflammatory cytokines IL-1β [26] and TNF-α [27]. The modulation of IL-8 secretion in gingival epithelial tissues during episodes of periodontitis [28, 29] and gingivitis [30, 31] is considered a key component for the maintenance of oral health [1, 32]. Therefore, in order to elucidate potential mechanisms by which the medicinal extract Sudantha (SUD) contributes to the promotion of gingival health and homeostasis, its immunomodulatory effects on gingival epithelial cell IL-8 production was determined.

This study found that SUD inhibited expression of the pro-inflammatory cytokine, IL-8, by gingival epithelial cells agonized with bacterial products (F. nucleatum cell wall extracts or live F. nucleatum) and host inflammatory mediators (IL-1β and TNF-α) in a dose-dependent manner. These data support the notion that one aspect of the efficacy of the Sudantha extract is in its ability to reduce excessive IL-8 secretion in response to both bacterial and host inflammatory signals.

Methods

Bacterial culture and crude cell wall

F. nucleatum ATCC 25586 was obtained from the Darveau laboratory bacterial collection and grown overnight in trypticase soy yeast broth (TYK) supplemented with 10 μg/mL hemin and 1 mg/mL menadione at 37 °C under anaerobic conditions (80% N2, 10% CO2, 10% H2). F. nucleatum crude cell wall samples were prepared as previously described [33] using a French cell pressure of 15,000 lb./in².

TIGK cell culture and infection

The immortalized human gingival keratinocyte cell line, TIGK, was generously provided by Dr. Richard J. Lamont2 and maintained in growth medium3 containing 25 μg/mL bovine pituitary extract, 0.2 ng/mL human recombinant epidermal growth factor, 0.4 mM calcium chloride, and 10% penicillin-streptomycin. Antibiotics were excluded for experiments with live bacteria.

TIGK cells were plated into 96-well plates at a density of 2 × 10⁴ cells/well and allowed to grow for 48 h until a confluence of approximately 90%. Test wells were stimulated in triplicate for 4 h with or without the extract (controls) at the indicated concentrations at 37 °C and 5% CO2 with the indicated ligands: live F. nucleatum bacteria at a multiplicity of infection (MOI) of 1:500 and F. nucleatum cell wall components, IL-1β 4 and TNF-α 5 all at 100 ng/mL.

Sudantha herbal extract

Sudantha (SUD) extract, provided by Dr. Devapiya Nugawela6 is a crude dark proprietary mixture of herbs that is incorporated into a commercially available toothpaste7. The formula of SUD is based on the recommendations of a specialist panel of Ayurvedic clinicians and contains a mixture of heartwood of cutch tree (Acacia

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3Keratinocyte-SFM, Thermo Scientific, Waltham, Massachusetts, USA
4Human IL-1β Recombinant Protein, eBioscience, San Diego, California, USA
5Human TNF-α Recombinant Protein, eBioscience, San Diego, California, USA
6Link Natural Products, Kapugoda, Sri Lanka
7Link Natural Products, Kapugoda, Sri Lanka
chundra Willd.), malabar nut leaf (Adhatoda vasica Nees.), Spanish cherry bark (Mimusops elengi L.), black pepper (Piper nigrum L.), pongam oil tree root (Pongamia pinnata(L.) Pirerre), Aleppo oak galls (Quercus infectoria Olivier.), clove (Syzygium aromaticum L.), myrobalan fruit (Terminalia chebula Retz.), and ginger (Zingiber officinale Roscoe). SUD is standardized and quality controlled by high performance liquid chromatography (HPLC). It was stored at 4 °C in the dark and freshly prepared to a stock concentration of 2 mg/mL in 0.2% ethanol. This stock concentration was then subsequently serially diluted two fold with TIGK growth medium to produce working concentrations of 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, 15.6 μg/mL, 7.8 μg/mL, 3.9 μg/mL, and 1.95 μg/mL.

Measurement of secreted IL-8 by enzyme-linked immunosorbent assay (ELISA)
After termination of the 4 h infection, culture supernatants were collected and diluted 2.5 fold in 1% bovine serum albumin in 1X PBS for determination of secreted IL-8 by standard sandwich ELISA. IL-8 monoclonal primary capture antibody and secondary biotin-labeled, detection-antibody were used for ELISA and detected with avidin-horseradish peroxidase enzyme (HRP) and tetramethylbenzidine (TMB) substrate. Optical densities were read at 450-570 nm on a microplate reader and concentrations were calculated from a standard curve using known concentrations of serially diluted recombinant human IL-8.

Half-maximal inhibitory dose (ID₅₀)
Half-maximal inhibitory dose (ID₅₀) was estimated from an experimentally derived dose-response curve for each concentration.

Cell viability
After removal of supernatant for IL-8 protein determination, cell viability was assessed using a fluorometric assay according to manufacturer protocols. In brief, 50 μl of growth medium was added to cells and followed by the addition of 50 μl of fluorometric reagent. Luminescence was measured after 10 min at room temperature using a microplate luminometer.

Statistical analysis
Student t tests were performed to determine significance of IL-8 responses by stimulated TIGK cells with and without extract. P values below 0.05 was considered significant (* P ≤ 0.05 ** P ≤ 0.01, *** P ≤ 0.001).

Results
Concentrations equal to or less than 250 μg/mL of SUD does not affect TIGK cell viability
The effect of SUD on TIGK cell viability was measured to find the optimal concentrations for further down-stream experimentation. Exposure to SUD for 4 h revealed that concentrations equal to or less than 250 μg/mL did not affect TIGK cell viability (Fig. 1). In contrast, concentrations of 500 μg/mL SUD showed cytotoxicity, reducing TIGK cell viability to 62%, and was excluded from further experimentation. Therefore, downstream characterization on the effects of SUD on modulation of host inflammatory mediators were performed with 250 μg/mL as the maximal dose. Furthermore, cell viability was examined concurrently with each experiment and showed similar results with no effect on TIGK cell viability.

SUD suppresses F. nucleatum cell wall extract induced IL-8 expression by gingival keratinocytes
F. nucleatum represents a common Gram negative species found in gingival plaque obtained from periodontally healthy and diseased [34–37] sites and have been shown to elicit a potent IL-8 response from gingival epithelial cells [24, 25, 38]. TIGK cells infected with 100 ng/mL of F. nucleatum cell wall extracts produced a potent

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1M801, Thermo Scientific, Waltham, Massachusetts, USA
2M802B, Thermo Scientific, Waltham, Massachusetts, USA
3VMax Microplate Reader, Molecular Devices, Sunnyvale, California, USA
4Human IL-8 Recombinant Protein, eBioscience, San Diego, California, USA
5GraphPad Prism 8, GraphPad Software, San Diego, California, USA
6CellTiter-Glo, G9241, Promega, Madison, Wisconsin, USA
7Centro LB 960, Berthold Technologies, Wildbad, Germany
IL-8 response that was suppressed by the addition of SUD in a dose dependent manner and that was not a result of cell death (Fig. 2). Suppression of IL-8 by *F. nucleatum* cell wall was first observed at 7.8 μg/ml SUD, while SUD at a range of 125–250 μg/ml worked optimally to reduce IL-8 expression down to basal levels without affecting cell viability.

**SUD suppresses live *F. nucleatum*-induced IL-8 expression by gingival keratinocytes**

To further characterize the anti-inflammatory effects of SUD against *F. nucleatum* induced IL-8 production, TIGK cells were infected with live bacteria at an MOI of 1:500. Similar to bacterial cell wall, live *F. nucleatum* at an MOI of 1:500, produced a potent IL-8 response (Fig. 3) greater than that observed with *F. nucleatum* cell wall alone and its expression was similarly dampened by SUD at concentrations of 1.95 μg/ml. This inhibition occurred in a dose dependent manner that brought IL-8 expression to basal levels of IL-8 with 125–250 μg/ml of SUD, similar to concentrations required to reduce IL-8 levels produced by *F. nucleatum* cell wall. Despite observable trends in IL-8 reductions at all test SUD concentrations, student t test confirmed the loss of statistical significance at 125–250 μg/ml of SUD, indicating significant reduction of IL-8 levels down to basal levels.

**SUD suppresses IL-1β and TNF-α host mediator induced IL-8 expression by gingival keratinocytes**

IL-1β and TNF-α are potent inflammatory cytokines reported to be involved in cellular proliferation, activation, and differentiation [39]. These host cytokines have been shown to induce IL-8 secretion from gingival epithelial cells [26, 27, 40, 41]. Consistent with previous reports of IL-1β and TNF-α induced expression of IL-8 by gingival keratinocytes, TIGK cells infected with 100 ng/ml of each cytokine for 4 h induced expression of IL-8 to 779 pg/ml and 3546 pg/ml, respectively (Fig. 4a and b). Inhibition of IL-8 occurred with the addition of SUD in a dose-dependent manner that was able to bring down IL-8
expression induced by IL-1β to 89 pg/ml and TNF-α induced expression to 235 pg/ml, almost down to control levels of 21–36 pg/ml IL-8. Statistical analysis revealed loss of statistical significance at concentrations of 62.5–250 μg/ml for IL-1β and 125–250 μg/ml for TNF-α.

Differential inhibition of agonist stimulated IL-8 expression by gingival keratinocytes

SUD was able to dampen both F. nucleatum and host cytokine induced gingival epithelial IL-8 inflammatory responses. Therefore, the 50% inhibitory dose (ID50), the concentration of the test compound required to inhibit the agonist induced cytopathogenic effect by 50% [42], was examined for preferential inhibitory effects between stimulation by bacterial products and host pro-inflammatory signals. Gingival epithelial cells secrete basal levels of IL-8 (Fig. 5) which was dampened to 50% expression by SUD at concentrations of 55.10 μg/ml. In contrast to mechanisms related to basal expression of IL-8, agonist stimulated IL-8 was dampened to 50% inhibitory levels at a much lower concentration. TNF-α stimulated IL-8 response was most sensitive to SUD with ID50 concentrations at 11.39 μg/ml SUD. While, IL-1 and live F. nucleatum required higher ID50 concentrations around 28.84 μg/ml and 25.77 μg/ml SUD respectfully. Therefore, these ID50 results demonstrate differential inhibitory effects of the extract between different IL-8 agonists.

Discussion

In general, the goal of treatment is to aid and enhance the inherent ability of the host’s innate defense mechanisms to restore compromised homeostasis. Consistent with this approach, the use of plant derived polyphenols as anti-inflammatory compounds have been intensely investigated [5–11] and shown to inhibit key mediators of the inflammatory cascade, including MAP kinases and nuclear transcription factors [5]. SUD, a proprietary mixture of natural herbs, has recently shown clinical success in its ability to restore and maintain gingival and periodontal health while providing antimicrobial activity [9, 19]. This manuscript describes the anti-inflammatory mechanisms exhibited by SUD against bacterial or host cytokine induced gingival epithelial cell IL-8 secretion and the potential benefits of reducing IL-8 in therapeutics.

IL-8 is a pro-inflammatory cytokine produced by a wide variety of cells including gingival epithelial cells, endothelial cells, gingival fibroblasts, neutrophils, monocytes, and phagocytes in response to bacterial invasion and plays a distinct role in neutrophil migration and activation [23, 39]. The importance of its role in neutrophil function has been recognized in oral health and disease [43, 44]. Irregular and uncontrolled expression of IL-8 contributes to neutrophil mediated-local-tissue-destruction (bystander damage) of periodontal tissues [1, 44]. Therefore, therapeutic approaches targeted towards the regulation of IL-8, and hence neutrophil homeostasis, would be greatly beneficial. Our study showed that TIGK cells stimulated with either live F. nucleatum or its cell wall components produced a potent IL-8 response which was suppressed by the addition of SUD in a dose dependent manner without affecting cell viability. Although there was reduction of IL-8 with the addition of SUD at all concentrations tested, significant reduction was shown at concentrations of 125–250 μg/ml when compared between SUD and control samples.

IL-1β and TNF-α are potent pro-inflammatory mediators secreted in response to bacteria and are associated with the pathogenesis and progression of periodontal disease [45–47]. They induce the upregulation of adhesion molecules on neutrophils and endothelial cells, stimulate the production of chemotactic molecules to induce neutrophil migration, and enhance inflammatory signals which potentiate inflammatory responses [48]. Consistent with this, gingival epithelial cells stimulated with IL-1β and TNF-α induced expression of IL-8 in this study, which was inhibited by SUD in a dose dependent manner. This inhibition was statistically significant at 62.5–250 μg/ml. Collectively, these data suggests that one mechanism behind the observed clinical efficacy of
SUD in clinical trials may be due to its ability to dampen neutrophil migration through the reduction of host or bacterially mediated IL-8 secretion.

It is noteworthy that different concentrations of SUD was required to reduce IL-8 response half maximally after stimulation by IL-1β and TNF-α. TNF-α induced IL-8 required 11.39 μg/ml of SUD, while IL-1β induced IL-8 required 2-fold this amount, 28.84 μg/ml of SUD. Gingival keratinocytes are reported to produce varying levels of basal IL-8 secretion [32, 49] (which may be important in the maintenance of healthy homeostasis) and SUD was required in significantly higher concentrations, to dampen the basal levels of IL-8 expression when compared to the agonist activated IL-8 secretion. Differences in SUD inhibitory concentrations required to inhibit between basal and host inflammatory modulator stimulated IL-8 suggests that agents in SUD demonstrate selective inhibition of different IL-8 stimulation pathways which may prove to be a useful tool to modulate host inflammatory responses. However, further work is required to better understand the potential selective action of SUD on IL-8 secretion and the possible mechanisms behind its clinical success.

Conclusions
Altogether, in vitro experiments of SUD on its ability to dampen the host immune response in relation to IL-8 stimulation by bacteria or host inflammatory mediators supports previously shown clinical beneficial effects of SUD for the maintenance of periodontal and gingival health. Specifically, this manuscript has demonstrated that at least one anti-inflammatory effect of SUD is the inhibition of gingival epithelial cell IL-8 secretion. Since, IL-8 is a potent neutrophil chemokine associated with gingivitis, the ability to dampen neutrophil migration represents a beneficial effect which may contribute to the efficacy observed in gingivitis clinical trials. However, additional experimentation is required to expand upon the potential of this extract to selectively modulate host inflammatory pathways without disturbing the cell intrinsic host inflammatory surveillance.

Abbreviations
HPLC: High performance liquid chromatography; HRP: Avidin-horseradish peroxidase enzyme; ID50: Half-maximal inhibitory dose; IL: Interleukin; MOI: Multiplicity of infection; SUD: Proprietary mixture of herbal extracts, peroxidase enzyme; TMB: Tetramethylbenzidine; TYK: Trypticase soy yeast broth

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Authors’ contributions
AMC and SB performed the experiments; AMC wrote the manuscript; SB, RP and SR edited the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
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