FINAL REPORT for:

SOOTHING SOLUTIONS LTD.

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Executive Summary

Soothing Solutions Ltd.'s Tonstix jelly pops were tested for wound healing and anti-inflammatory properties in a human pharyngeal epithelial cell model (Detroit 562) by Shannon ABC. Initially, Detroit 562 cells were treated with Tonstix jelly pop samples at various concentrations to assess any potential cytotoxicity and to determine the most appropriate concentrations to use in the wound healing (scratch wound assay) and anti-inflammatory assays**.**

Cytotoxicity assay

Cytotoxicity evaluation of the Tonstix jelly pop samples was assessed in the Detroit 562 cells using the Resazurin assay to determine the acceptable concentration of the product to introduce into the cell line for further bioassays. It was found that all concentrations of Tonstix jelly pop samples tested were non-cytotoxic to the cells.

Anti-inflammatory assay

As none of the concentrations of Tonstix jelly pops samples were toxic to the cells, the highest concentration (0.2g/mL) and a diluted concentration (0.05g/mL) was selected to treat the Detroit 562 cells for testing in an anti-inflammatory assay. Lipopolysaccharide (LPS) was the inflammatory stimulus and the inflammatory marker quantified using the ELISA method is interleukin-6 (IL-6). Tonstix jelly pop samples were found to reduce IL-6 levels at both concentrations tested.

Scratch wound assay

The effect of Tonstix jelly pop samples on cell migration/wound healing of Detroit 562 cells was investigated using the scratch wound assay. Concentrations of Tonstix jelly pop samples used for the assay were (0.2g/mL) and (0.05g/ mL). FBS (10%, 5% and 2.5%) were used as positive controls. Tonstix jelly pop samples exhibited wound healing activity undigested and digested at 0.05g/mL concentration.

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Background

Soothing Solutions Ltd.'s popular medicinal free and naturally soothing Tonstix jelly pops underwent *in vitro* cellular bioactivity assays using Detroit 562 pharyngeal cells. The aim of this study was to investigate Tonstix jelly pops potential anti-inflammatory activity against an established pro-inflammatory cellular insult of lipopolysaccharides (LPS)(1) and potential wound healing properties using the scratch wound assay which measures the migration and repair of a cell monolayer after a set scratch or 'wound' is introduced. The more of the scratch area recovered by new growth of cells over a set period of time indicates faster or improved 'healing'. Fetal bovine serum (FBS) is an established positive control for this assay.

Materials and Methods

Cell line and media

Detroit 562 cells were purchased from American Type Culture Collection (ATTC). Detroit 562 cells were thawed 2 weeks before testing. Once thawed, approximately $1x10^6$ cells were seeded into a T-75 flask and grown in a humidified incubator at 37°C, 5% CO_2. Cells were passaged when the monolayer reached 70-80% confluence. Detroit 562 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Biosciences Ltd, cat# 11054020-500ML), 10% foetal bovine serum (FBS) (Gibco™, cat#10091148) and 2mM solution of L-Glutamine (Sigma-Aldrich, cat# 59202C-100mL).

Sample preparation

6.8959g of Tonstix jelly pop sample was weighed and underwent an artificial oral digestion using Simulated Salivary Fluid (SSF) as outlined in Table 1. To mimic oral digestion, extracts were mixed with 8 ml of SSF. 50 µl of 0.3 M $CaCl_2(H_2O)_2$ and 1.95 mL of H_2O were combined and thoroughly mixed. The Tonstix jelly pop sample was made up to 20 ml by adding SSF. Final concentration of Tonstix jelly pop samples was 6.8959g in 20 mL i.e., 0.4 g/ mL. Along with artificially oral digested samples, the same concentrations of undigested samples (dissolved in media) were used in each of the assays also.

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Constituent	Volume [mL]
0.5 M KCl	4.53
0.5 M KH2PO4	1.11
1 M NaHCO3	2.04
MgCl2(H2O)6	0.15
0.5 M (NH4)2CO3	0.018
Total volume **7.848 ml**	
Adjust to pH 7 using 6 M HCl	
Make up to a total volume with water 120 ml	

Table 1. Simulated Salivary Fluid (SSF) composition

Cytotoxicity assay

The effect of test samples on the cell viability of Detroit 562 cell line was determined using the resazurin assay. The resazurin assay is widely used to quantify the total number of viable cells in an *in vitro* culture. The assay principle is based on the reduction of oxidized non-fluorescent compound resazurin, which is blue in colour, by the mitochondrial respiratory chain of viable cells to a red fluorescent dye called resorufin. The resorufin dye production is directly proportional to viable cell count. It has a fluorescence excitation/emission wavelength of 530-560/ 590 nm. After the 24-hr period of test samples treatment, resazurin reagent (Sigma-Aldrich, cat#R7017-5G), prepared by dissolving 7.5mg resazurin sodium salt in 50 ml PBS, was added to each well and incubated for 2 hours at 37°C. Relevant fluorescence values were then measured using a Varioskan Lux (Thermo Scientific) plate reader.

Anti-inflammatory assay

Detroit 562 cells were seeded at a density of $2x10^5$ cells per well in 24 well plates. The plates were incubated in a humidified incubator at 37˚C, 5% CO_2 overnight. The cells were then treated with Tonstix jelly pop samples for 24 hours. 0.001µM dexamethasone (Sigma-Aldrich, cat#D4902-25MG) was used as positive control. After the sample treatment, cells were stimulated for 24 hours with 1µg LPS (Sigma-Aldrich, cat# L2654-1MG). Following treatment

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incubations, supernatants were removed from each well of the 24 well plates and assayed for IL-6 using the Human IL-6 ELISA kit (DY206, R&D Systems).

ELISA was performed in accordance with manufacturer's instructions. To summarize, Capture Antibody was added to the wells of a clear 96 well plate and incubated overnight at room temperature. The next day, the plate was washed twice with 400 µL Wash Buffer and blocked with Reagent Diluent for 1hr at room temperature. The wells were washed twice with 400 µL Wash Buffer again before standards and diluted samples were added and incubated for 2 hours at room temperature. The plate was washed twice more with 400 µL Wash Buffer before Detection Antibody was added and incubated for 2hr at room temperature. The plate was washed again twice followed by the addition of Streptavidin-HRP and incubation at room temperature for 20 min. The plate was washed a final two times before Substrate Solution was added for 20 min at room temperature. Stop Solution was added and absorbance was read at 450 nm and 540 nm using a Varioskan Lux Plate reader (Thermo Scientific).

Scratch wound assay

The effect of Tonstix jelly pop samples on the migration/wound healing of Detroit 562 cells was investigated using the scratch wound assay. Briefly, Detroit 562 cells were seeded in 24 well plates at a density of $2x10^5$ cells per well. The plates were incubated in a humidified incubator at 37˚C, 5% CO_2 to allow for attachment in a confluent monolayer. On the day of the scratch wound assay, the cells were removed from the humidified incubator. A linear scratch in the cell layer of each well was performed using a sterile 200µL pipette tip at the centre of each of the wells in the plate. The cell media was removed, ensuring no cell debris remained in the well, before replacing with fresh media containing the Tonstix jelly pop samples (0.2g/mL and 0.05g/ mL). 10% FBS was added to the relevant wells. Each sample was tested in triplicate in the assay plate to account for cell or scratch variability. Each well was analysed at time points 0hr and 6hr for Detroit 562 cells with photomicrographs obtained for each well at each timepoint using an Optika Vison Pro microscope attachment and software.

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Scratch closure percentage were calculated from scratch area using the following formula:

$$Scratch\ closure = \frac{A_0 - A_t}{A_0} \cdot 100\%$$

A_0: scratch area at the first time point, A_t: scratch area at the current time point.

Results

1. Cytotoxicity assay

Cell viability was performed using the Resazurin assay. Detroit 562 cell viability was measured after 24 hrs treatment with Tonstix jelly pop samples (Figure 1).

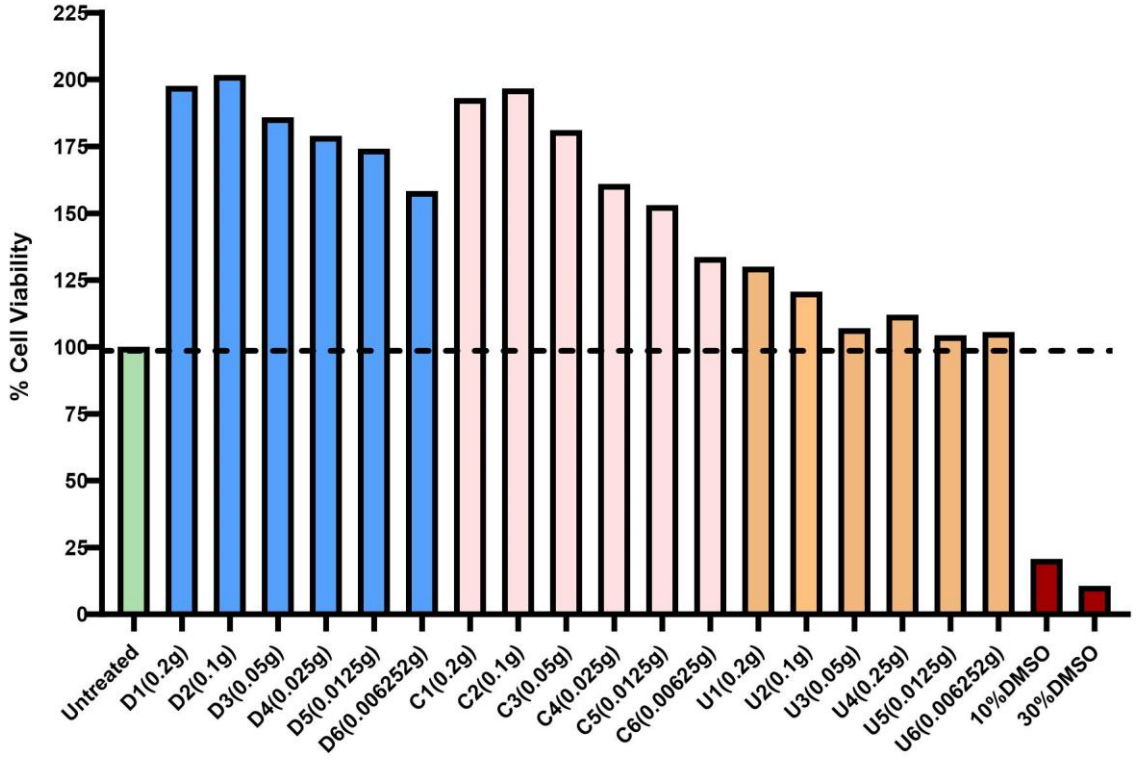


Figure 1. Cell viability measurement using Resazurin assay. Cell viability levels post 24 hr treatment. Fluorescence at 560nm/590nm following addition of Resazurin reagent. All the Tonstix samples tested were non cytotoxic to Detroit 562 cells. Artificially oral digested samples were D1-D6, Undigested samples were U1-U6 and Digested control were C1-C6. The positive controls (10% DMSO and 30% DMSO) severely reduced cell viability. N=1 for this assay.

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2. Anti-inflammatory assay

IL-6 production of Detroit 562 cells was determined using a human IL-6 ELISA kit.

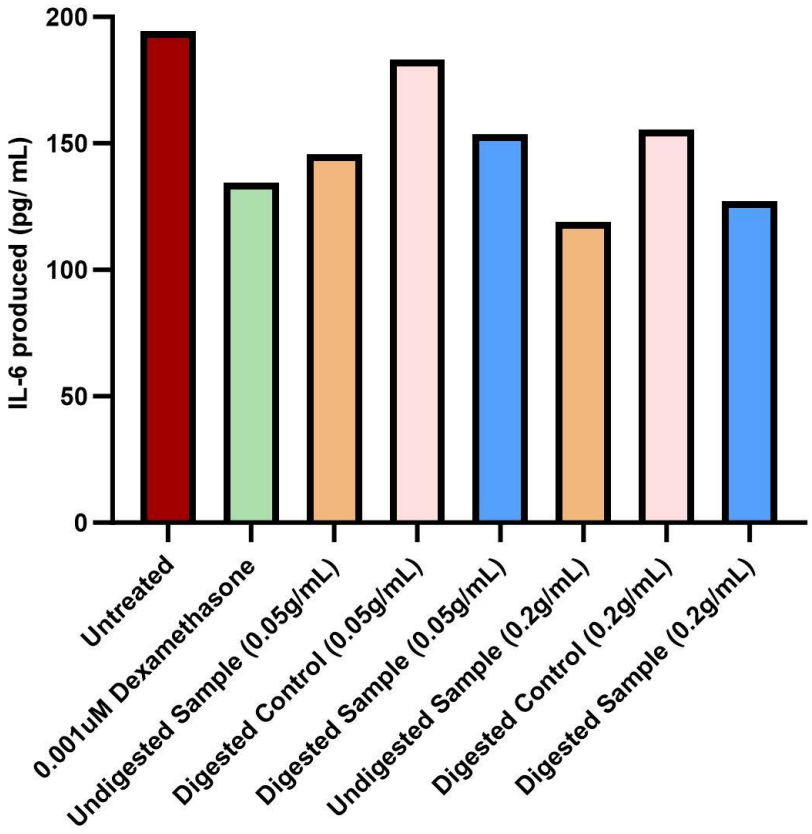


Figure 2. IL-6 production of Detroit 562 cells after a 24 hr LPS 1µg stimulus for each. Positive control was 0.001µM dexamethasone. The positive control, digested and undigested sample of Tonstix jelly pop samples reduced the production of IL-6 in Detroit 562 cells compared to Untreated (LPS stimulation alone). N=1 for this assay.

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3. Scratch wound assay

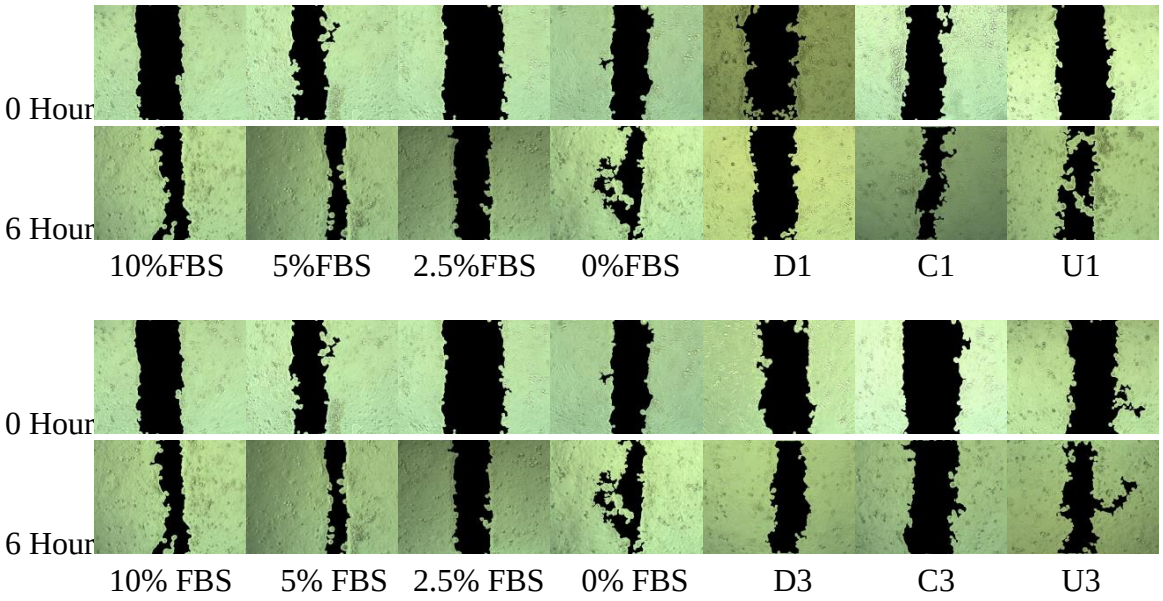


Figure 3a. Images of scratch wound areas. The positive controls were 2.5%, 5% and 10 % FBS containing DMEM cell culture media and the vehicle control was 0% FBS containing DMEM cell culture media. D1 (0.2g/mL) and D3 (0.05g/ mL) were the digested samples, C1 (0.2g/mL) and C3 (0.05g/ mL) were the digested control samples and U1 (0.2g/mL) and U3 (0.05g/ mL) were the undigested samples of Tonstix jelly pops. When compared with digested control of lower concentration (C3) the digested sample at lower concentration (0.05g/ mL) showed an increase in wound closure.

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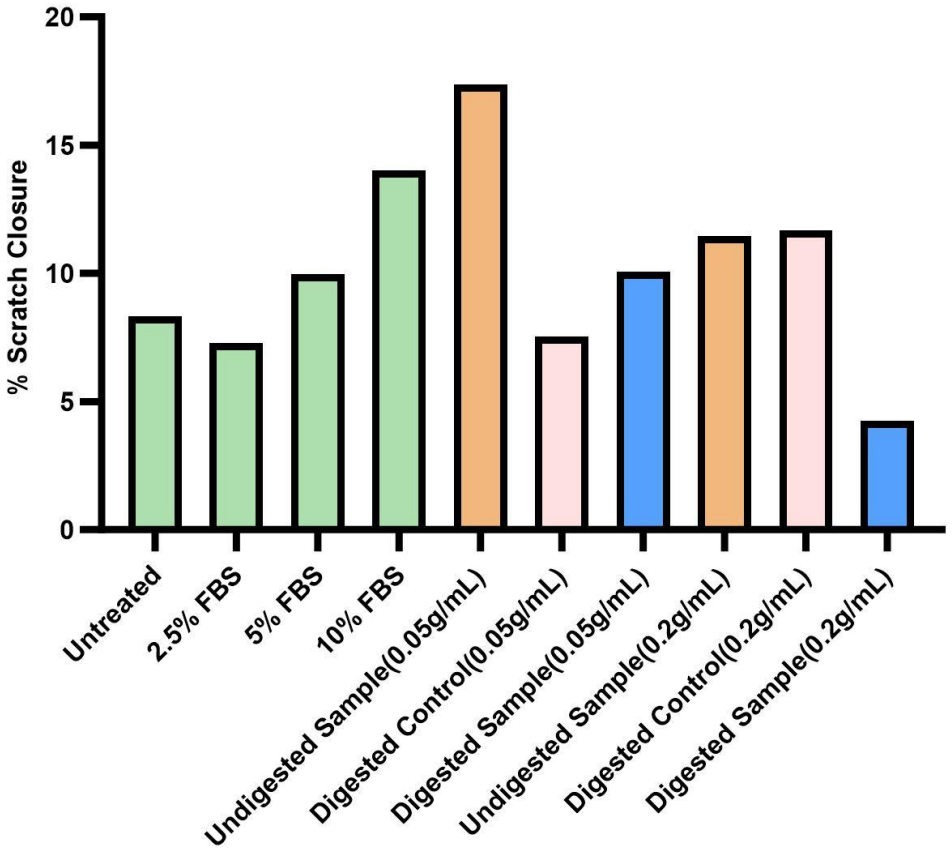


Figure 3b. Percentage values of scratch area closure. The positive controls were 2.5%, 5% and 10 % FBS containing DMEM cell culture media and the untreated control was 0% FBS DMEM cell culture media. When compared with digested control, the digested sample at the lower concentration (0.05g/ mL) showed an increased wound closure percentage.

Discussion and Conclusions

- It was found that none of the concentrations of digested and undigested samples of Tonstix jelly pops were toxic to Detroit 562 cells after 24-hour treatment **(Figure 1)**.
- The SSF itself appears to confer a proliferative effect as seen by the significant increase in cell viability in all samples containing it.
- Tonstix jelly pop samples reduced LPS-induced IL-6 levels at both concentrations tested (0.2g mL and 0.05g/ mL), a finding that is linked to anti-inflammatory activity **(Figure 2)**.
- When compared with the digested control, the digested sample at lower concentration of 0.05g/ mL showed an increased wound closure percentage, a finding linked to potential wound healing activity **(Figure 3a & Figure 3 b)**.

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