Ficus septica exudate, a traditional medicine used in Papua New Guinea for treating infected cutaneous ulcers: in vitro evaluation and clinical efficacy assessment by cluster randomised trial

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Abbreviations: DPP (dual path platform, syphilis screen and confirm); ELISA (enzyme-linked immunosorbent assay); FCS (foetal calf serum); GM-CSF (granulocyte-macrophage colony-stimulating factor); IL-6 (interleukin-6), LPS (lipopolysaccharide); MMP (matrix metalloproteinase); NMR (nuclear magnetic resonance); PBS (phosphate buffered saline); TNF-α (tumour necrosis factor-α); Tx (treatment).

ABSTRACT

Background and objectives: Infected cutaneous ulcers are a major health problem for children living in rural areas of Papua New Guinea. The inaccessibility of affected populations and lack of access to basic healthcare, makes a local plant-based therapy an attractive treatment option. We assessed *Ficus septica* exudate in biological assays relevant to wound healing. We then carried out a clinical trial to determine the exudate's efficacy in healing small cutaneous ulcers compared with chlorhexidine cetrimide cream, and soap and water washing.

Methods: Pre-clinical *in vitro* assessment of the exudate was carried out using assays to monitor the pro-inflammatory responses of M1 macrophages and neutrophils, antibacterial assays using known ulcer pathogens, an Ames test for mutagenicity and LC-MS chemical analysis of the exudate. An open label cluster-randomised clinical trial was performed, enrolling participants from three different clusters with skin lesions less than 1cm in diameter. Each cluster comprising 50 participants was randomly assigned to one of three treatment arms namely topical exudate, topical chlorhexidine with cetrimide cream, and standard care (soap and water treatment), all administered daily for 2 days. The primary outcome was clinical healing/improvement measured at days 7 and 14, assessed by three dermatologists using blinded photographs. Primary analysis was non-inferiority of *F. septica* treatment based on the risk difference for healing/improvement.

Results: *In vitro*, the exudate which is rich in the alkaloid ficuseptine, was found to be non-mutagenic whilst also inhibiting pro-inflammatory responses and exhibiting antibacterial activity. When administered to participants enrolled in the clinical trial, no significant differences were observed between the healing efficacy of *F. septica* exudate and the two comparators (chlorhexidine cream and soap/water treatment). At day 14, but not at day 7, the efficacy of *F. septica* exudate for healing/improving the ulcers was non-inferior to chlorhexidine or water/soap treatment.

Conclusions: *F. septica* exudate is non-mutagenic and has both bactericidal and anti-inflammatory properties. When applied topically to small cutaneous ulcers, the exudate has a healing effect that is non-inferior to chlorhexidine and standard treatment with soap and water at day 14. Our findings, which should be confirmed in larger clinical trials, have important public health implications.

Introduction

Infected cutaneous ulcers are a painful condition that are common in amongst children in Papua New Guinea. The ulcers present as single or multiple non-purulent skin lesions showing epidermal loss, and are chronic, generally lasting more than 2 weeks (Noguera-Julian et al. 2019). They are believed to develop from a small scratch or trauma, which subsequently becomes infected. In rural tropical regions, cutaneous ulcers are most prevalent in children, with infection rates that may range from 5 to 15% (Noguera-Julian et al. 2019). Although accurate figures for Papua New Guinea are lacking, our own observations suggest that most children living in rural villages either have active ulcer infections or show scars from previous ulcers. As an estimated 6.4 million people (80% of Papua New Guinea's population) live in rural areas (Schmidt Mueller et al., 2020), cutaneous ulcers represent a significant area of unmet clinical need. Furthermore, the unusual circumstances in Papua New Guinea, in which relatively large and scattered rural populations live in highly inaccessible rainforest villages has prevented the development of an effective aid post network, and as a result, in these areas, conventional treatment options for cutaneous ulcers are extremely limited. For these populations, early treatment of small, infected lesions with a locally available plant antiseptic is preferable to late treatment with antibiotics in a hard-to-reach clinic and may, therefore, represent a valuable treatment option. Acknowledging the limited access to medicines in rural areas, the Papua New Guinea standard treatment guidelines for children recommend the use of soap and water to prevent skin disease and ulcers (Paediatric Society of Papua New Guinea, 2016); however, evidence for this approach from clinical trials is currently lacking.

The microbiology of cutaneous ulcers in Papua New Guinea is a developing research area; the aetiology of cutaneous ulcers may differ, with *Treponema pallidum* subsp *pertenue* (the causative agent of yaws disease) and *Haemophilus ducreyi* (the causative agent of chancroid) suggested to be causative agents (Mitja et al. 2014; Noguera-Julian et al. 2019). Earlier work dating back to the 1980s that used culture-based identification methods had identified *Fusobacterium ulcerans* as a potential pathogen (Aadrians and Shah 1988; Lupi et al. 2006) whereas another study carried out in the highlands of Papua New Guinea identified β-haemolytic *Streptococci*, *Staphylococcus aureus*, *Corynebacterium diphtheriae* and *Corynebacterium haemolyticum* as potential wound pathogens (Montgomery 1985). More recent metagenomics-based studies carried out in the Solomon Islands, identified *H. ducreyi* as a prominent pathogen (Marks et al., 2014), and further application of metagenomics to yaws-like ulcers in Lihir Island (Papua New Guinea) revealed a range of bacterial species including *H. ducreyi*, *Streptococcus pyogenes and Streptococcus dysgalactiae* (Griesenauer et al. 2021; Noguera-Julian et al. 2019).

An additional aspect to be considered is the role of excessive inflammation in these ulcers. Although little is known regarding the role of inflammation in small cutaneous ulcers in Papua New Guinea, extensive research carried out on venous ulcers, diabetic ulcers, and pressure sores in high income

countries, has revealed a correlation between the chronic nature of the ulcers and persistent or dysregulated inflammation (Zhao et al. 2016). In these types of ulcers, pro-inflammatory neutrophils and M1 macrophages, which predominate during early dermal healing, persist in the ulcers, resulting in wounds perpetually remaining in the early inflammatory phase (Eming et al. 2007). The neutrophils and macrophages are responsible for elevated levels of pro-inflammatory cytokines, such as IL-1 and IL-6, and TNF-α. These in turn increase the expression of matrix metalloproteinases (MMPs), which degrade extracellular matrix components within these wounds (Barrientos et al. 2008; Moseley et al. 2004). Additionally, fibroblasts present in nonhealing wounds display impaired proliferative and migratory responses as a consequence of enhanced cellular senescence within chronic wound environments (Wall et al. 2008). Thus, topical exudate treatments that eradicate wound infections, reduce the subsequent inflammatory responses, or stimulate fibroblast proliferation and keratinocyte healing responses may reduce the chronic non-healing nature of cutaneous ulcers (Moses et al. 2020b).

F. septica Burm.f. (Moraceae), is a tree or shrub with yellowish exudate, and has a distribution that includes Taiwan, Malesia (including New Guinea and the Bismark Archipelago), Australia (Northern Queensland) through to Vanuatu (Berg Corner, E.J.H. 2005). *F. septica* exudate has traditionally been used as a topical treatment for cutaneous ulcers in Papua New Guinea and our own ethnobotanical observations in the Kaulong speaking area of New Britain reveal the exudate is still used as a topical treatment for cutaneous ulcers (Baumgartner et al. 1990). The exudate is obtained by breaking off the figs and allowing the exudate to drip from the broken peduncle onto the surface of infected cutaneous ulcers in the same manner as a topical antiseptic. The tree is commonly found in secondary forest near villages and is therefore a highly accessible treatment option. Extensive phytochemical isolation from stems, roots, and leaves of *F. septica* has yielded a wide variety of phenanthroindolizidine alkaloids, ficuseptine and ficuseptines A-N (Damu et al. 2005, 2009; Kubo et al. 2016; Ueda et al. 2009; Wu et al., 2002). In addition, vanillic acid, (5-acetyl-2-hydroxyphenyl)-β-D-glucopyranoside, the coumarins umbelliferone and esculin, the flavonoids genistin and kaempferitrin, squalene, and uracil have been isolated from the leaves (Wu et al., 2002). *F. septica* has antimicrobial activity which has been attributed to the alkaloids ficuseptine and antofine (Baumgartner et al. 1990; Prescott et al. 2015). Furthermore, two alkaloids reported from *F. septica*, tylophorine and ficuseptine-A, both inhibit nitric oxide production in lipopolysaccharide (LPS)/interferon-γ -stimulated macrophages, without causing significant cytotoxicity (Yang et al. 2006).

In the present study, we investigated the suitability of *F. septica* exudate as a low-cost treatment option for small cutaneous ulcers in rural areas of Papua New Guinea. *F. septica* is particularly suitable as a first-line treatment because it is commonly found growing near areas of human habitation. Our study includes both an *in vitro* assessment of the exudate's suitability as a topical ulcer treatment, as well as a clinical trial to determine its efficacy.

Materials and methods

Collection and preparation of plant material

Permission for collection and issuing of export licences for botanical specimens was provided by the National Herbarium of Papua New Guinea. Two exudate samples were collected along with corresponding voucher specimens; one sample for *in vitro* pre-clinical testing and the other sample for the clinical trial. The exudate was collected by precisely replicating the traditional method which was observed first-hand during previous ethnobotanical fieldwork (Prescott et al., 2012). The exudate was harvested by picking the figs and allowing a drop of exudate to form on the broken peduncle (stalk); the exudate droplet was then touched onto the inside edge of a 2ml cryovial and the process repeated until the vial was filled with exudate. The sample for *in vitro* testing was collected in Umbi Village, West New Britain in March 2017. A voucher specimen (T.A.K.P. 164) from the same tree is lodged at the herbarium, Royal Botanic Gardens, Kew. To prepare this exudate sample for *in vitro* work, the exudate was centrifuged at 13,400 g, and then filtered through a 0.02 µm PTFE syringe filter under sterile conditions and stored at -20 °C. Subsequent lyophilisation and weighing of the centrifuged and filtered exudate sample used for the *in vitro* pre-clinical experiments revealed it to have a concentration of 147.43 mg exudate metabolites per ml of exudate.

The sample for the clinical trial was collected directly into plastic vials using the same method but without centrifugation and filtration. The exudate was collected in November 2019 from two separate tree specimens in Lihir Island and Kokopo, East New Britain, vouchers for each respectively, (T.A.K.P. 176) and (T.A.K.P. 177) are lodged at Kew and the Forest Research Institute, Lae, Papua New Guinea. The two exudate samples were pooled together to make a single sample and stored briefly at 5 °C before beginning the clinical trial. A portion of the clinical trial exudate sample was centrifuged and filtered in the same way as the *in vitro* pre-clinical sample and, after lyophilisation and weighing, was found to have a concentration of 158.48 mg/ml. For all samples, botanical identifications were carried out by comparison with authentic herbarium reference material.

Liquid chromatography–mass spectrometry (LC–MS) analysis of exudate sample

Liquid chromatography-mass spectrometry (LC-MS) was carried out with a ThermoFisher Scientific 'Vanquish' UHPLC system coupled to a Thermo Scientific 'LTQ-Orbitrap XL' hybrid linear ion trap-orbitrap mass analyser fitted with an electrospray ionisation (ESI) source. Samples (5 µL) were injected into a Phenomenex Luna C18 column (150 x 3 mm internal diameter, 3 µm particle size) and eluted at 0.4 ml/min and 30 °C using a linear gradient of MeOH, H_2O, and MeCN with 1% formic acid (0:90:10–90:0:10 v/v over 60 min). MS^1 spectra at 30,000 resolution were recorded by Fourier transform mass spectrometry (FTMS) in both positive and negative modes in the *m*/*z* range 125–1800. Simultaneously, the linear ion-trap (ITMS) recorded low resolution MS^1,MS^2, and MS^3 spectra (*m*/*z* 125-1800).

For quantification of ficuseptine in exudate samples, a sevenfold serial dilution of a pure ficuseptine standard was prepared using a sample obtained previously (Prescott et al. 2015). A seven point dilution series from 500µg/ml to 7µg/ml ficuseptine was prepared and then analysed by LC-MS along with a five hundredfold dilution of the exudate samples. For the clinical trial exudate sample, a dilution was prepared from the pure unfiltered unrefined sample. Quantification was achieved by measuring UV peak area at 285nm and then interpolating the readings from exudate samples to the linear range of the ficuseptine standard curve (r2=0.999).

Ames tests with Salmonella typhimurium TA98 and TA100 strains

The 24-well plate bacterial reverse mutation test was performed following standard methods (Escobar et al., 2013; Maron and Ames, 1983; Mortelmans and Zeiger, 2000). Briefly, 5 µL of solvent, positive control or test item were added to wells of a 24-well plate containing minimal glucose agar. A mixture of 5 µL of overnight bacterial culture, 25 µL of sodium phosphate buffer or S9 mix (Molecular Toxicology Inc, USA), and 100 µl of molten 0.6% agar were maintained at approximately 50 °C and supplemented immediately with 0.05 mM biotin and histidine. Once the agar had solidified, plates were inverted and incubated for 3 days at 37 °C. After incubation, wells of all plates were examined both macroscopically and microscopically for evidence of cytotoxicity or precipitates and any other effect relevant to the interpretation of the test. Microscopic examination was used to check the condition of the histidine-requiring micro-colonies that make up the 'background lawns'. Revertant (his+) colony numbers were scored manually.

Antibacterial assays

Bacterial strains: *S. aureus* (NCTC 6571), *S. dysgalactiae* (NCTC 13762), *S. pyogenes* (NCTC 8198), *C. diphtheria* (NCTC 11397) and *F. ulcerans* (NCTC 12112) were grown at 37 °C. Logarithmic phase cultures of *S. aureus*, S. *dysgalactiae* and *S. pyogenes* were grown aerobically in brain heart infusion (BHI) broth (Sigma-Aldrich, Poole, UK). *C. diphtheria* was grown in BHI under microaerophilic conditions (candle jar) and *F. ulcerans* was grown anaerobically in fastidious anaerobic broth (Lab M, Heywood, UK).

The disc diffusion assays were carried out as described previously (Moses et al. 2020a, b). Positive control discs contained 15 µL 10 % w/v povidone iodine (Vetasept) or 0.2 % chlorhexidine. The minimum inhibitory concentration (MIC; lowest concentration of the test compounds to inhibit visible microorganism growth after overnight incubation) and minimum bactericidal concentration (MBC; lowest concentration of the test compounds to prevent microorganism growth after subculturing) were performed as follows. Sterile filtered exudate samples were diluted in broth to obtain desired exudate concentrations alongside serial dilutions of 0.2% w/v chlorhexidine, the starting dilution being 5% (100 µg/ml). The bacterial inoculum was adjusted for each organism, to yield a cell concentration of 5×10^4

colony forming units (CFU)/ml, and 95 µL of inoculum was added to microtitre plate wells (Nunc, Thermo Fisher Scientific, Loughborough, UK). A total volume of 5 µL exudate solution was then added to wells and incubated overnight. After overnight incubation, plates were examined for visible microorganism growth and the lowest concentration of the test compound at which no visible bacterial growth occurred was recorded as the MIC. Samples were then removed from each well, serially diluted and plated out on Columbia blood agar plates for bacterial viability counting of *S. aureus, S. dysgalactiae, S. pyogenes, C. diphtheria* or plated on fastidious anaerobic agar plates for *F. ulcerans* enumeration (Southern Group Laboratory, Corby, UK). The MBC was recorded as the lowest concentration of the test compound that prevented bacterial growth on subculturing.

Isolation of neutrophils and macrophages from whole human blood

Neutrophils and non-polarised macrophages were isolated from whole blood, based on the original method of Moseley et al. (2003), as described recently (Moses et al. 2020a, b). Whole blood (in 20 ml aliquots) was collected from healthy, human volunteers (age range 20-40 years).

Pro-inflammatory cytokine release by human neutrophils and M1 macrophages

Pro-inflammatory cytokine quantification was carried out, as described previously (Moses et al. 2020a, b). Briefly, isolated neutrophil or macrophage cultures ($5x10^5$ cells/ml) were established in the absence and presence of non-cytotoxic dilutions of *F. septica* exudate. Il-6 and TNF-α levels in cell culture supernatants were determined using standard ELISA procedures. Experiments were performed on n=3 independent occasions, with data expressed as pg/ml.

Dermal fibroblast and keratinocyte proliferation

Primary dermal fibroblasts derived from normal skin were purchased from ATCC (Teddington, UK), (HaCaTs) were obtained from the German Cancer Research Centre (Heidelberg, Germany). Cell proliferation assays were carried out, as described previously with experiments performed on n=3 independent occasions, and data expressed as a % versus untreated controls (Moses et al., 2020).

Clinical trial participants and study setting

Following the *in vitro* assessment, we conducted a prospective, open label, cluster-randomized controlled trial at three neighbouring schools with 150 students aged 9 to 18. The trial was carried out in East New Britain, Papua New Guinea, between Nov 1, and Dec 15, 2019. Each school which corresponded to a single cluster was randomly assigned to one of the three treatment arms after screening by a physician.

Investigators subsequently assessed all children within the 9 to18 age range in each school, starting from the lowest grades. All children with skin lesions of less than 1 cm in diameter were assessed for eligibility. Exclusion criteria were the need for antibiotic therapy or a known history of hypersensitivity

or allergy to the investigated product. Eligible children were subsequently offered the opportunity to enrol in the study until the 50 designated spaces per treatment arm had been filled.

Children who agreed to enrol in the study provided verbal assent, and their parents or legal guardians gave signed written consent for participation, according to standard protocols implemented and described previously (Gonzalez-Beiras et al. 2017). The protocol was approved by the Research and Ethics Committee of the School of Medicine and Health Sciences, University of Papua New Guinea and is registered at ClinicalTrials.gov with Identifier: NCT04453124. The study followed the guidelines of the Declaration of Helsinki and Tokyo for humans.

Randomization and intervention

Schools were randomly assigned to receive either *F. septica* exudate, chlorhexidine with cetrimide cream (Savlon) antiseptic cream (Novartis Consumer Health, UK) or recommended standard care (washing the ulcer with soap and water). All interventions were administered twice, at baseline (day 0) and again at day 1, by the research team.

To apply the exudate, 50 μL exudate was pipetted onto the tip of the index finger of a volunteer nurse wearing surgical gloves. The exudate was then applied directly to the ulcer surface taking care to ensure the exudate was evenly distributed on the ulcer. New gloves were used for each patient. The Savlon cream (cetrimide 0.5%w/w and chlorhexidine gluconate 0.1% w/w) was applied in the same way, with 200mg of the cream applied to the glove. The standard care, soap and water wash was carried out by a nurse according to the Papua New Guinea health guidelines using locally available sodium palmate based "WasWas" brand of soap.

Trial procedures

Before the intervention, a physician carried out a structured assessment of the skin, arms, face, and legs of each participant (baseline assessment). Information was recorded from each enrolled participant on a case report form (CRF) specifically designed for the study. A *Treponema* DPP syphilis screen and confirm assay (Chembio Diagnostic Systems) was carried out at baseline (pre-treatment) according to manufacturer's instructions as described previously (Ayove et al. 2014), along with a lesional swab, taken using a sterile cotton swab and stored in DNA/RNA shield (Cambridge Biosciences). Patients testing positive with the DPP assay and showing no clinical resolution after two weeks were treated with azithromycin, as described previously (Mitja et al. 2014). The physician selected one skin lesion that met the eligibility criteria and recorded its location in the CRF for further assessments. A photograph of each lesion was taken at baseline (before treatment) and at days 7 and 14 post-treatment. Adhesive labels printed with QR codes to identify each patient were applied next to the ulcers when pictures were taken.

Photographs of each ulcer were blinded by digitally removing the identification code present in the photograph. The photos were sent to three dermatologists, who graded the ulcers according to level of improvement at 7 and 14 days relative to baseline into the following categories: worse, unchanged, improved, and healed. Discordant grading was resolved between the three dermatologists using expert opinion from co-author S.L.W, (London School of Hygiene and Tropical Medicine, UK).

PCR of ulcer swabs

DNA was extracted using the DNA Advance Magnetic Bead Extraction Kit (Beckman Coulter, UK) following a modification of the manufacturer's protocol. Briefly, 200 µL of extraction buffer comprising 173 µL lysis buffer with 5 µL of lysozyme 20 mg/ml, 7 µL proteinase K (40 mg/ml) and 5 µL 1 M DTT, were added to each sample and incubated for 1 h at 56 °C. Samples were mixed by pipetting with 100 µL of Bind1 Buffer, followed by 170 µL of Bind2 Buffer. A magnet was applied to bind the beads and the solution removed. Three washes of 340 µL 80% ethanol were applied, each time removing the tubes from the magnet and reapplying them to remove liquid. Samples were eluted into 100 µL of Elution Buffer. Samples were quantified by fluorimetry using the Qubit dsDNA High Sensitivity Kit (Invitrogen, Carlsbad, USA) and also quantified on a Nanodrop spectrophotometer (GE Healthcare, Amersham, UK) to provide an indication of DNA purity. A260/A280 ratios were >1.70. Primers were designed and wet-lab validated for specificity, sensitivity, linearity over 7 log, and efficiency >95% by qStandard (UK). For each target, a standard of 107 copies was prepared from purified PCR products and serially diluted 10-fold to 10 copies. Primer sequences for the single copy Pol A gene of both bacterial species were as follows; *T. pallidum* F: aatggtgtggtcgcgtttgat R: gaagagaaacgtccggaacaataag, *H. ducreyii* F: cgaatatcgcgcataaattgctga R: ttagctgaggtaacgagtgaaca. Primers for human (YWHAZ) gDNA were: F: acttttggtacattgtggcttca R: ccgccaggacaaaccagtat. For qPCR 1 ng DNA was used per reactions and qPCR was performed as described previously (Lowe et al., 2019).

Statistical analysis

Results of the *in vitro* analyses were summarized as mean ± standard error of the mean (SEM) of n=3 independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) with post-Dunnett's test for multiple comparisons. The significant threshold was set at an alpha-error of 0.05.

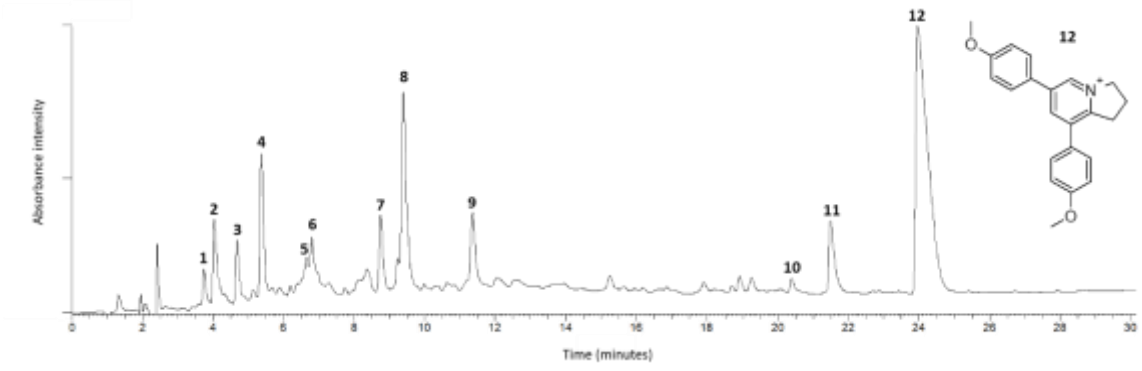
Clinical data were described using frequencies and percentages of each category of lesion progression: worse, unchanged, improved, and healed. The primary endpoint of the clinical assessment was the percentage of participants with an improved/healed lesion within each study arm. We assessed the non-inferiority of *F. septica* exudate vs. chlorhexidine and *F. septica* exudate vs. standard care (soap and water) based the absolute risk difference between arms using the standard normal approximation to obtain the confidence interval (CI) of the risk difference (Tang et al., 2014); non-inferiority was

considered when the lower boundary of the 95% CI of the risk difference was greater than -10 percentage units. All analyses were performed in R 3.6.3 and package dani (Quartagno 2020).

Results and discussion

LC-MS analysis reveals the alkaloid ficuseptine is a major component of the exudate

Previous phytochemical work on the leaves, roots, and stems of *F. septica* has yielded a variety of phenanthroindolizidine alkaloids (Damu et al. 2005). Our previous work on the related species *Ficus botryocarpa* identified the alkaloid ficuseptine as the principle active antibacterial compound (Prescott et al. 2015). Our LC-MS analysis of *F. septica* exudate also showed ficuseptine as a major component (Figure 1.). We also detected ficuseptine C, seco-dehydroantofine, and ficuseptine D along with a variety of caffeoylgalactaric acid and sinapoylgalactaric acid isomers. Their identifications were based on comparison with mass spectrometric and UV spectra data reported in the literature (Baumgartner et al. 1990; Damu et al. 2005; Kubo et al. 2016; Ueda et al. 2009). Both exudate samples presented the same major peaks and, in both cases, the antibacterial alkaloid ficuseptine was the major peak. As ficuseptine has already been demonstrated to be an active antibacterial principle we decided to quantify concentrations of the compound in both the clinical trial exudate sample and the sample used in the pre-clinical investigations. Quantitation using a ficuseptine standard revealed the sample for *in vitro* pre-clinical testing contained 53.9mg/ml ficuseptine whereas the clinical trial sample (un-processed as used in the trial) contained 23.3mg/ml ficuseptine, (r2=0.999).



Peak	Identification
1-3	caffeoylgalactaric acid or isomer
4	ficuseptamine C
5	caffeoylgalactaric acid or isomer
6-9	sinapoylgalactaric acid and isomers
10	seco-dehydroantofine
11	ficuseptine D
12	ficuseptine

Fig. 1. Photometric Diode Array (PDA) chromatogram of *F. septica* exudate sample used in the clinical trial. Identity of peaks were confirmed based on their mass spectrometry fragments and UV spectra.

F. septica exudate is antibacterial towards wound pathogens known to colonise cutaneous ulcers in Papua New Guinea

Application of a topical antiseptic to the ulcer surface can reduce bacterial bio-burden and concomitant bacterial toxin production allowing wound progression and healing. We carried out disc diffusion assays and broth micro-dilution assays with non-fastidious bacterial pathogens that have been previously isolated or detected from cutaneous ulcers in Papua New Guinea (Montgomery 1985; Aadrians and Shah 1988; Noguera-Julian et al. 2019). The topical wound antiseptics chlorhexidine and povidone iodine were included as positive control agents. A disc diffusion assay was used as it measures the ability of a test substance to diffuse across an aqueous surface. This is conceptually similar to the *in vivo* application of the *F. septica* exudate to the ulcer surface, where diffusion across the aqueous wound milieu is important to ensure elimination of bacterial bio-burden. In the disc diffusion assay (Table 1.) *S. aureus* was the most sensitive organism. The exudate shows greater activity than a positive control comprising 0.2 % w/v chlorhexidine which is the concentration of chlorhexidine used in antiseptic formulations. The exudate is slightly less active than the 10 % w/v povidone iodine sample. In the broth microdilution assay the exudate was most active against *S. aureus* and *C. diphtheriae* and least active with *S. pyogenes* where full inhibition of growth was not observed. The two different exudate samples showed similar levels of activity in their MIC values but differed more in the MBC values (Table 2). For both exudate samples, the MIC and MBC values were well within the concentrations of plant exudate that would be present on the ulcer surface where the volume of exudate is generally greater than the volume of wound exudate present. This suggests the exudate could exert a bactericidal activity at the ulcer surface.

	S. aureus	*S. pyogenes*	*F. ulcerans*
F. septica exudate	5.9	5.5	2.2
Chlorhexidine	3.3	4.5	2.0
Povidone iodine	6.9	6.0	1.8

Table 1. Zones of inhibition (radius, in mm) with *S. aureus, S. pyogenes* and *F. ulcerans* in a disc diffusion assay. Each 6 mm paper disc was treated with 15 µL *F. septica* exudate (*in vitro* sample), 15 µL 10 % w/v povidone iodine or 15 µL 0.2 % w/v chlorhexidine, before applying to agar plates seeded with *S. aureus* (NCTC 6571), *S. pyogenes* (NCTC 8198) and *F. ulcerans* (NCTC 12112). Povidone and chlorhexidine results were obtained as part of a multiple sample experiment carried out in parallel and reported previously (Moses et al. 2020a, b).

	Exudate sample 1 MIC	Exudate sample 1 MBC	Exudate sample 2 MIC	Exudate sample 2 MBC	CHX MIC	CHX MBC
C. diphtheriae	461 (0.31)	921 (0.63)	495 (0.31)	991 (0.63)	1 (0.04)	3 (0.16)
F. ulcerans	921 (0.63)	7372 (5)	991 (0.63)	3962 (2.5)	2 (0.08)	6 (0.31)
S. pyogenes	-	-	-	-	0.4 (0.02)	0.4(0.02)
S. aureus	230 (0.16)	7372 (5)	247 (0.16)	991 (0.63)	2 (0.08)	13 (0.63)
S. dysgalactiae	1843 (1.25)	3686 (2.5)	991 (0.63)	7924 (5)	2 (0.08)	6 (0.31)

Table 2. Minimum inhibitory concentrations and minimum bactericidal concentrations for two *F. septica* exudate samples and the positive control chlorhexidine. Values outside brackets indicate MIC and MBC values expressed as μg/ml. Values in brackets indicate MIC and MBCs as % v/v dilutions where the undiluted exudate is 100% and the chlorhexidine (CHX) dilution is % v/v of a 0.2% w/v stock solution. Exudate sample 1 is the sample used for pre-clinical *in vitro* testing, exudate sample 2 is the clinical trial sample. For *S. pyogenes* both exudate samples gave partial but incomplete inhibition at the test concentrations used.

The exudate is not mutagenic in Ames tests with Salmonella typhimurium TA98 and TA100

The exudate was tested for mutagenic activity in a bacterial reverse mutation assay. *S. typhimurium* LT2 bacteria strains TA98 and TA100 were used as indicator organisms to detect frame shift and base substitutions respectively. The assay uses standardised protocols described previously (Maron and Ames 1983; Mortelmans and Zeiger 2000; Escobar et al. 2013). Rat liver S9 homogenate was used to check for metabolic activation of the exudate, and the known mutagens sodium azide and 2-aminoanthraene were used as positive controls. In accordance with standard procedures, a positive response was considered when the minimum mean number of revertants per well increased by threefold over the corresponding solvent control. The results (Figure 2.) show that while the known mutagenic positive controls show increases above threefold for each strains as expected, with the exudate there were no consistent increases in numbers of revertant colonies for either strain including in the presence of S9 metabolic activation.

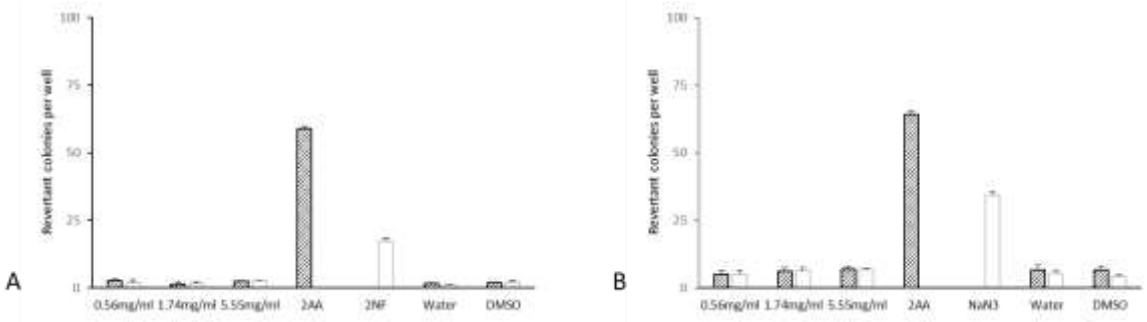


Fig. 2. The exudate does not induce mutations in the TA98 Salmonella strain (A) or the TA100 strain (B) when tested at concentrations up to 0.56mg/ml (3.703% v/v). Shaded bars indicate S9 metabolic activation, unshaded without S9 activation. Figures in mg/ml indicate concentration of *F. septica* exudate. 2AA (0.37µg/ml 2-aminoanthracene), 2NF (0.37µg/ml nitrofluorene), NaN_3 (0.19µg/ml sodium azide). Error bars indicate the standard error of the mean (SEM).

The exudate stimulates proliferation of keratinocytes but not, dermal fibroblasts

Both fibroblasts and keratinocytes play an important role in wound healing, with both cell types required for deposition of extracellular matrix components such as collagens, proteoglycans and hayaluronin (Moseley et al. 2004). However, fibroblasts present in chronic wounds exhibit reduced proliferative and migratory capabilities, due to the increased onset of cellular senescence within chronic wound environments, while keratinocytes also show reduced migratory responses (Wall et al. 2008; Pastar et al. 2014). We examined the effect of the exudate on fibroblasts and keratinocytes. Figures. 3A and 3B show that although the fibroblasts showed no significant increases in proliferation in response to the exudate (all $p > 0.05$), the keratinocytes exhibited significantly increased proliferation at 24 h post-treatment ($p < 0.05$ at 15ng/ml exudate).

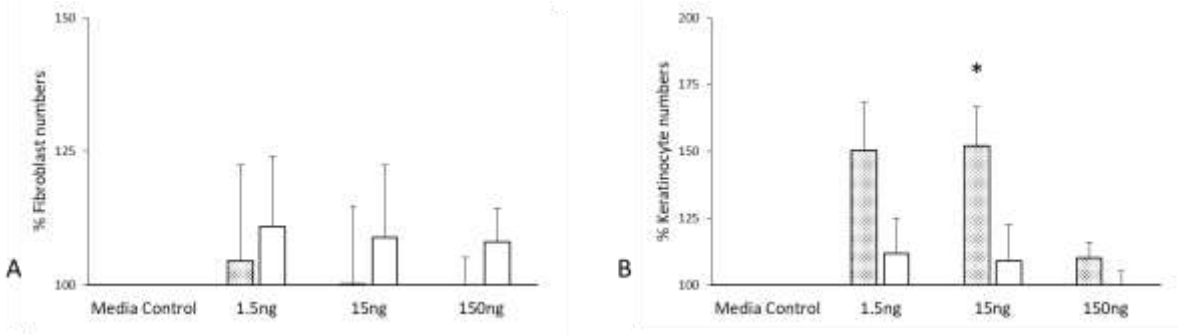


Fig. 3. Exudate effects on dermal fibroblast proliferation (A) and keratinocyte proliferation (B) at 24 h (shaded) and 72 h (unshaded), relative to treatment with media only. Error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments. *P* values *$p<0.05$.

The exudate completely down regulates cytokine expression from M1 macrophages and neutrophils

We next looked at the effect of the exudate on the pro-inflammatory responses of M1 macrophages and neutrophils that are known to reside within chronic wounds. Excessive inflammatory responses from these cell types in chronic wounds is known to result in elevated levels of IL-1, IL-6, and TNF-α, which in turn results in upregulation of MMP expression and degradation of extracelluar matrix (Barrientos et al. 2008; Moseley et al. 2004). Primary cells obtained from whole human blood were used as they

closely resemble the status of these cells *in vivo*. For the neutrophils, cells treated with and without LPS were included as controls to show the extent of the inflammatory response. For macrophages, control cells treated with or without GM-CSF were included to show polarisation occurred as expected. Figure 4A-4D shows that for both neutrophils and M1 macrophages, the exudate potently downregulates IL-6 or TNF-α secretion. The neutrophils are more sensitive to the exudate than the macrophages, with complete knock down of IL-6 or TNF-α levels occurring at 1.5μg/ml exudate (0.001% v/v) compared with 15μg/ml for IL-6 from macrophages. Interestingly, very low amounts of the exudate 0.15 μg/ml caused a significant increase in IL-6 expression from the macrophages, suggesting the exudate may elicit both pro-inflammatory and anti-inflammatory effects depending on the concentrations used.

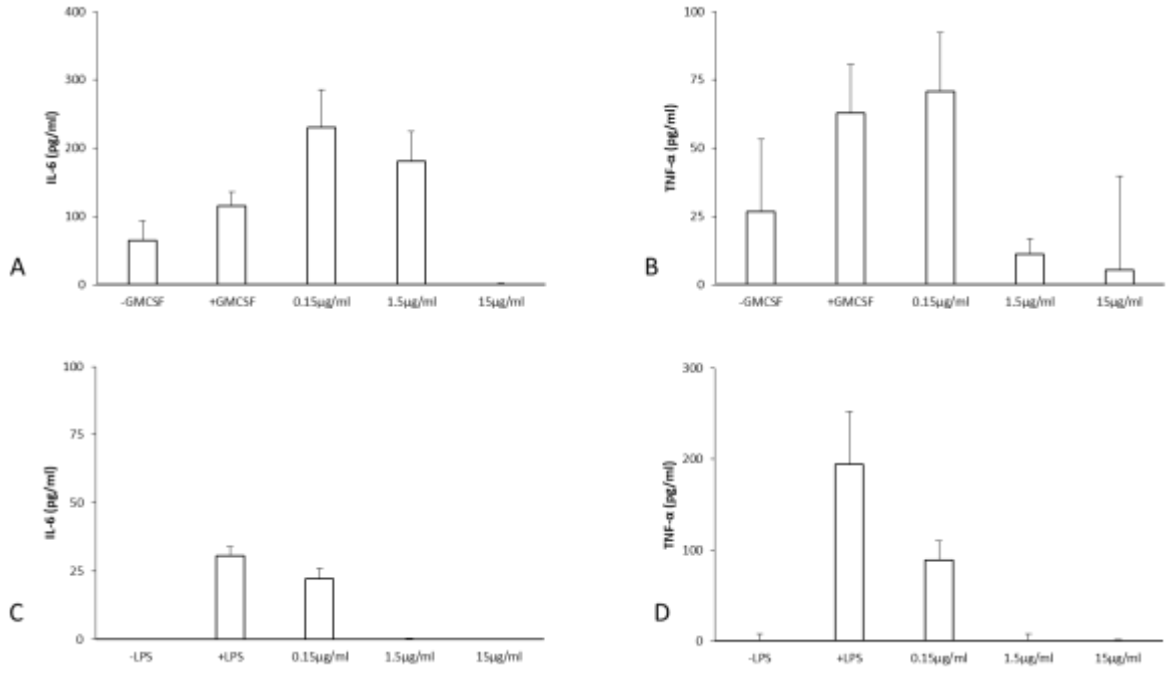


Fig. 4. Top two graphs show the effect of the exudate on IL-6 (A) and TNF-α (B) secretion by M1 macrophages. All conditions included stimulation with GM-CSF, apart from the –GM-CSF controls as indicated. Controls –GM-CSF and +GM-CSF show responses of pre-macrophages and polarised M1 macrophages respectively, each in the absence of exudate. Concentrations indicated in μg/ml are GM-CSF-stimulated cells treated with *F. septica* exudate at the specified concentrations. The bottom two graphs show the effect of the exudate on IL-6 (C) and TNF-α (D) secretion by neutrophils. All conditions included stimulation with LPS, apart from the -LPS control as indicated. Controls -LPS and +LPS indicate cell responses with and without LPS, in the absence of exudate. Bars with concentrations indicated in μg/ml are LPS-stimulated cells with *F. septica* exudate at specified concentrations. For all graphs, error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments. *P* values **p*<0.05, **p<0.01, ***p<0.005.

A clinical trial of the exudate shows similar levels of healing to chlorhexidine and cetrimide cream

The clinical assessment included 150 children aged 9 to 18 randomized at school level to treat skin ulcers with a standard care consisting of soap and water (control), *F. septica* exudate, or chlorhexidine with cetrimide cream (Savlon) antiseptic cream. This age range was chosen as the ulcers are particularly prevalent in children. All participants received two treatment rounds; 134 attended at least one follow-up visit and were therefore included in the non-inferiority analysis (Figure 5).

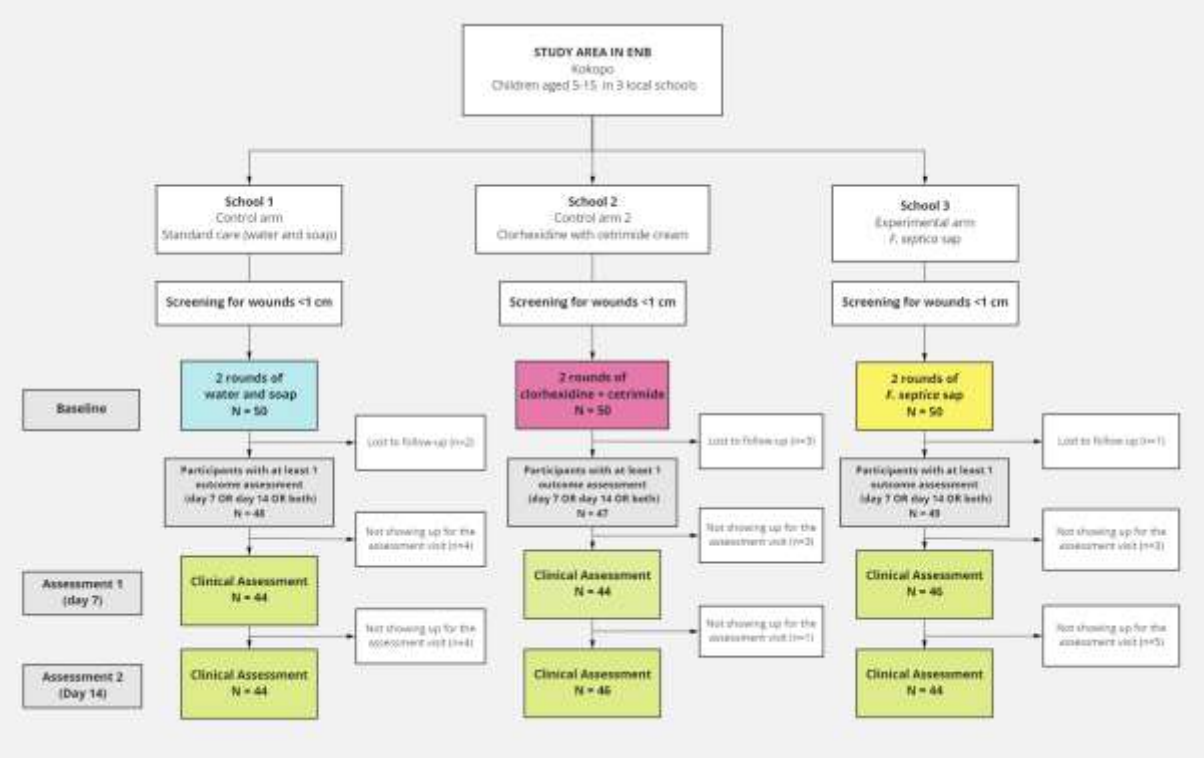


Fig. 5. A consort flow diagram of the clinical trial. The three treatment arms were as follows: recommended standard care (washing with soap and water), chlorhexidine and cetrimide cream and *F. septica* exudate. The clinical assessment was carried out on blinded photographs of ulcers. ENB**:** East New Britain

Figure 6 shows the result of the clinical assessment of skin lesions at days 7 and 14. The assessment was performed by expert dermatologists using blinded ulcer photographs. The dermatologists categorized the ulcers into one of four healing states, namely: worse, unchanged, improved, and healed. On both day 7 and 14, most participants had either improved or healed skin lesions, irrespective of the type of treatment (Figure 6). Accordingly, the risk difference estimate revealed no significant differences between treatments in the likelihood of improvement/healing at day 7 or 14 (Figure 7).

According to the pre-set noninferiority margin, *F. septica* exudate was non-inferior to chlorhexidine cream or standard care with water and soap at day 14; the noninferiority criterion was not met at day 7.

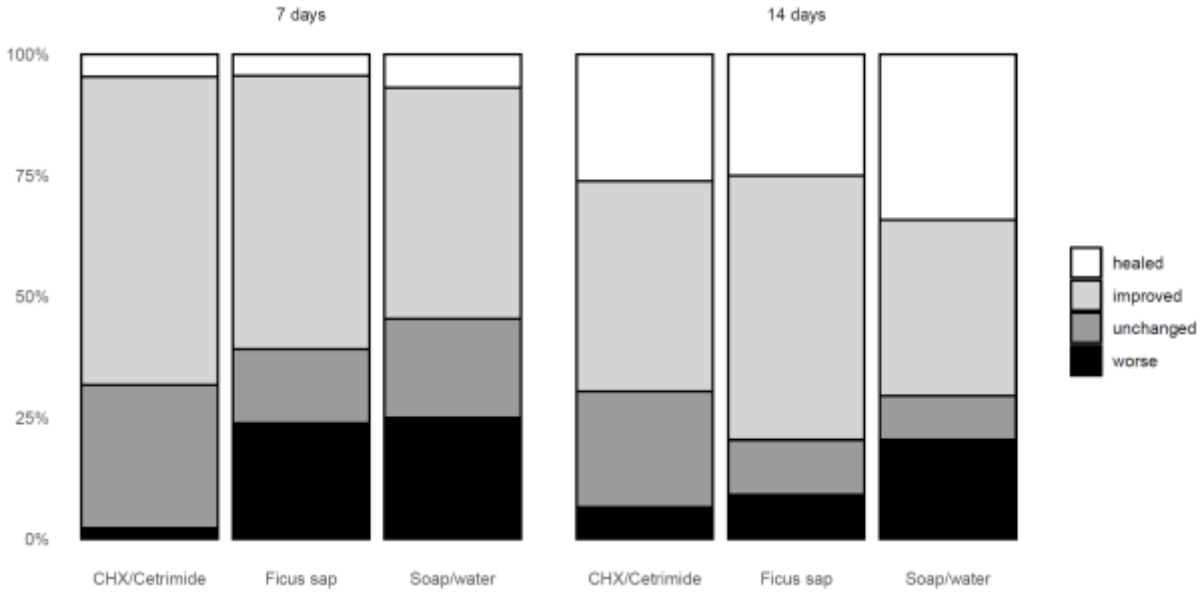


Fig. 6. Proportion of participants within each of the categories of lesion progression (i.e., worse, unchanged, improved, and healed) at each assessment. Results are presented for each treatment arm and assessments at days 7 and 14.

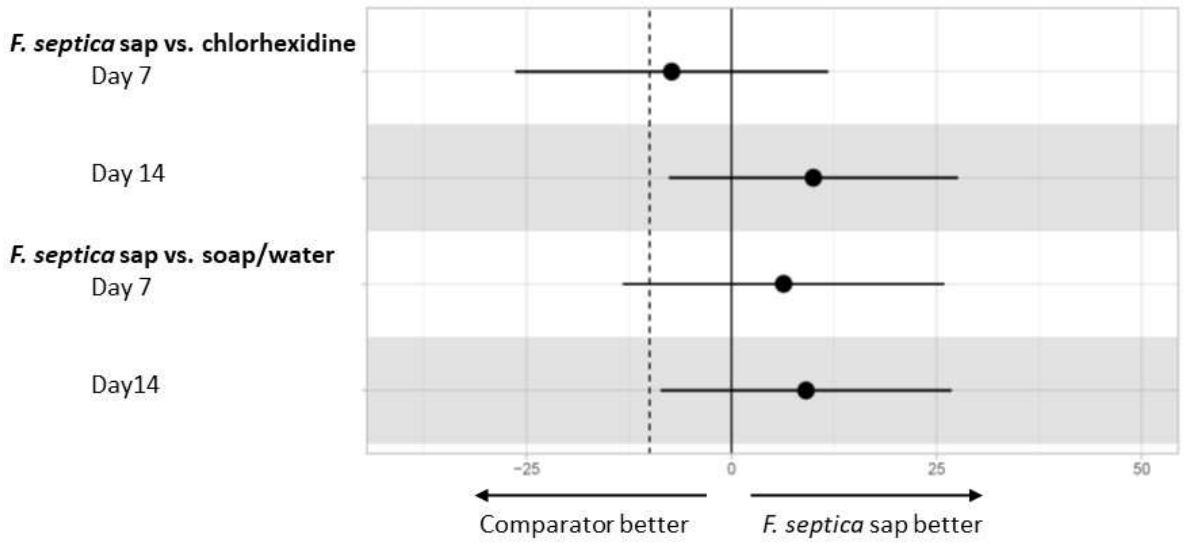


Fig. 7. Absolute risk difference for improvement/healing between treatment with *F. septica* exudate and the two comparators: chlorhexidine with cetrimide cream (Savlon) antiseptic cream and recommended standard care (washing the ulcer with soap and water). Dots and horizontal lines represent the absolute risk difference and the 95% confidence interval (CI). The dashed line shows the non-inferiority margin for the lower boundary of the 95% CI (-10 percentage points).

H. ducreyi and *T. pallidum* subspecies *pertenue*, are two bacterial species that have been detected in previous work on larger yaws-like ulcers in the South Pacific (Noguera-Julian et al. 2019). PCR diagnostics were carried out on wound swabs taken from each patient to establish if these bacterial species were also present in the smaller, less than 1cm diameter ulcers under investigation in this study. Participants in the three study arms showed a high proportion of *H. ducreyi* and a low proportion of *T. pallidum* subspecies *pertenue*, which is the causative agent of yaws disease (Table 4). We also used a DPP finger prick DPP syphilis screen and confirm assay, to detect active yaws cases, similarly revealing a low proportion in each treatment arm.

	F. septica exudate	CHX/cetrimide	Soap and water
H. ducreyi (PCR)	92%	98%	100%
T. pallidum (PCR)	0%	4%	4%
T. pallidum DPP	0%	4%	6%

Table 4. Proportion of patients in each treatment arm with PCR positive ulcers for *H. ducreyi*, *T. pallidum* subspecies *pertenue* as well as proportions of patients with active yaws infection determined with a DPP syphilis screen and confirm assay.

Conclusions

This is the first study to investigate the potential of *F. septica* as a first-line treatment for cutaneous ulcers and is the first clinical trial study of a plant medicine for infected cutaneous ulcers of the type commonly found in the South Pacific. The *in vitro* results presented here demonstrate that *F. septica* exudate exhibits bactericidal activity against both Gram-positive and Gram-negative bacterial pathogens that are known to infect ulcers in Papua New Guinea. The exudate also showed strong inhibition of pro-inflammatory responses from neutrophils and M1 macrophages, and it was non-mutagenic when tested in Ames assays looking for frameshift and base substitution mutations. Chemical analysis of the exudate revealed the presence of a variety of different alkaloids, but with the known antibacterial alkaloid ficuseptine the dominant major component. Thus, from the *in vitro* data, *F. septica* exudate exhibits potentially useful traits for a topical treatment for infected cutaneous ulcers of the type commonly encountered in Papua New Guinea. Furthermore, *F. septica* is a common plant species and

is often found growing in close proximity to villages in Papua New Guinea. The physical characteristics of the exudate are well suited to its traditional use as a topical treatment for cutaneous ulcers. Its sufficiently fluid to transfer from the tree to the ulcer but still viscous enough to adhere to the ulcers. Highly potent stimulation of keratinocytes was also observed but caution should be applied when considering this effect in an *in vivo* context as the topical application of the plant exudate would result in much higher concentrations of plant exudate at the ulcer surface and the *in vitro* results do not show stimulation of keratinocytes at higher concentrations.

Next, we conducted a cluster-randomized trial to investigate the healing effect of *F. septica* exudate on small skin lesions. To assess the microbiology of the ulcers, PCR diagnostics were carried out on lesion swabs, revealing the presence of both *H. ducreyi* and *T. pallidum* subspecies *pertenue*. Of the two species, *H. ducreyi* is by far the most prevalent, with detection rates ranging from 92% to 100% in the three treatment arms. Although previous work has applied metagenomics and PCR to large, yaws-like ulcers (Mitja et al. 2014), this is the first study to specifically examine small ulcers, less than 1cm diameter. The results provide further confirmation of the association of *H. ducreyi* with infected cutaneous ulcers in the South Pacific and suggest that *H. ducreyi* infection occurs early on in small ulcers rather than being acquired later on as a secondary infection of larger ulcers. The results also demonstrate the potential for small cutaneous ulcers to act as a gateway for yaws disease by providing a route into the body to establish a systemic infection.

The clinical trial compared healing outcomes of the exudate with chlorhexidine antiseptic cream and the standard treatment (soap and water) which is recommended in Papua New Guinea treatment guidelines. The analysis showed no significant differences between treatments. More interestingly, *F. septica* exudate was non-inferior to both chlorhexidine cream or soap and water at day 14. Our findings, which should be confirmed in a larger clinical trial, have important public health implications in preventing complications of skin ulcers. Although chlorhexidine cream is available in pharmacies in Papua New Guinea, a substantial section of the Papua New Guinean population lack ready access to pharmacies, aid posts or clinics. Furthermore, even soap and water treatment using clean water is not readily available to patients living in remoter areas of the country. Therefore, educational programs for self-care of small skin lesions using *F. septica* exudate, might form part of a low-cost health intervention for treating ulcers in remote areas of Papua New Guinea. Larger clinical trials with an adequate sample size are warranted to permit the inclusion of *F. septica* treatment into clinical guidelines and public health strategies.

Author contributions

John Deli (Investigation), Camila González-Beiras (Project administration; Investigation), Georgia S. Guldan (Supervision), Ryan Moseley (Supervision), Rachael L. Moses (Investigation), Jordanna

Dally (Investigation), Fionnuala T. Lundy (Formal analysis; Supervision), Marc Corbacho-Monne (Formal analysis), Stephen L Walker (Formal analysis), Maria Ubals (Formal analysis), Dan Ouchi (Formal analysis) Rui Fang (Formal analysis), Marie Briggs (Formal analysis) Robert Kiapranis (Project administration), Martha Yahimbu (Project administration), Oriol Mitjà (Conceptualization; Supervision), Thomas A.K. Prescott (Conceptualization; Investigation; Funding acquisition)

Conflict of interest

The authors declare no conflict of interest. The Savlon antiseptic cream used in this study was purchased from a high street pharmacy and the manufacturer did not provide funding for this study. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy

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