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Topical application of the bee hive protectant propolis is well tolerated and improves human diabetic foot ulcer healing in a prospective feasibility study

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ABSTRACT

Aims: Propolis is a naturally occurring anti-inflammatory bee derived protectant resin. We have previously reported that topically applied propolis reduces inflammation and improves cutaneous ulcer healing in diabetic rodents. The aim of this study was to determine if propolis shows efficacy in a pilot study of human diabetic foot ulcer (DFU) healing and if it is well tolerated.

Materials: Serial consenting subjects ($n = 24$) with DFU ≥4 week's duration had topical propolis applied at each clinic review for 6 weeks. Post-debridement wound fluid was analyzed for viable bacterial count and pro-inflammatory MMP-9 activity. Ulcer healing data were compared with a matched control cohort of $n = 84$ with comparable DFU treated recently at the same center.

Results: Ulcer area was reduced by a mean 41% in the propolis group compared with 16% in the control group at week 1 ($P < 0.001$), and by 63 vs. 44% at week 3, respectively ($P < 0.05$). In addition, 10 vs. 2% ($P < 0.001$), then 19 vs. 12% ($P < 0.05$) of propolis treated vs. control ulcers had fully healed by weeks 3 and 7, respectively. Post-debridement wound fluid active MMP-9 was significantly reduced, by 18.1 vs. 2.8% week 3 from baseline in propolis treated ulcers vs. controls ($P < 0.001$), as were bacterial counts ($P < 0.001$). No adverse effects from propolis were reported.

Conclusions: Topical propolis is a well-tolerated therapy for wound healing and this pilot in human DFU indicates for the first time that it may enhance wound closure in this setting when applied weekly. A multi-site randomized controlled of topical propolis now appears to be warranted in diabetic foot ulcers.

1. Introduction

Foot ulceration secondary to diabetes occurs in up to one quarter of people with diabetes (Bentley & Foster, 2007) and it is the commonest cause of lower limb amputation (Boulton, Vileikyte, Ragnarson-Tennvall, & Apelqvist, 2005). Diabetes increases the risk of lower extremity amputation by 10 to 20 times (Wrobel, Mayfield, & GE, 2005) and the estimated cost to the US healthcare system of diabetic foot ulceration and related amputations is more than $10.9 billion annually (Shearer, Scuffham, Gordois, Oglesby, & Tobian, 2003). Thus diabetic foot ulceration is a cause of significant morbidity and financial burden.

The delayed wound healing observed in diabetic foot disease is attributable to a variety of factors including peripheral arterial disease, peripheral neuropathy, foot deformity and secondary bacterial infection (Cavanagh, Lipsky, Bradbury, & Botek, 2005). Furthermore, the wound microenvironment in diabetes is abnormal and pathogenic factors lead to delayed ulcer closure, and suboptimal volume of granulation tissue formation with abnormal extra-cellular matrix (ECM) composition (Falanga, 2005; Pradhan et al., 2011). Specifically, it has been proposed that a persistent inflammatory infiltrate also associated with bacterial colonization in the wound contributes to delayed healing in diabetes (Falanga, 2005).

Propolis is a resinous bee-hive product consisting of plant materials that are initially collected on the hind legs of worker bees. The material is then masticated, salivary enzymes are added and mixed with wax to

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produce propolis (Bankova, De Castro, & Marcucci, 2000; Bufalo et al., 2013; Wagh, 2013a). Its most biologically active fractions are flavanoids and esters of caffeic acid (Banskota et al., 2002; Russo, Longo, & Vanella, 2002). Propolis has multiple properties that make it an attractive agent for treatment of diabetic foot ulcers, including being anti-inflammatory (Grunberger et al., 1988), anti-oxidant (Fonseca et al., 2011; Nagaoka et al., 2003; Talas et al., 2014) and anti-microbial (Gekker, Hu, Spivak, Lokensgard, & Peterson, 2005; Mirzoeva, Grishanin, & Calder, 1997) especially anti-bacterial (Astani et al., 2013; Scheller, Tustanowski, Kurylo, Paradowski, & Obuszko, 1977), in its actions. Furthermore, propolis component caffeic acid, has potent activity to inhibit the pro-inflammatory proteinase, matrix metalloproteinase-9 (MMP-9), and MMP-9 is known to be increased in diabetic foot ulcers (Jin et al., 2005; Ladwig et al., 2002; Liu et al., 2009).

We have previously published in a preclinical, diabetic rodent model of full thickness cutaneous wound healing, that a single application of topical propolis normalized ulcer closure rate and reduced persistent neutrophil infiltration and elastase activity (McLennan et al., 2008). In humans, propolis has been described as a useful topical treatment for ulcers (Wagh, 2013b). It is considered to have a low side-effect profile (Gallo et al., 2014; Rajpara et al., 2009; Sforcin & Bankova, 2011) and is approved in many countries for treatment of ulcers and abrasions, being sold over the counter in many parts of the world including in Australasia (Wagh, 2013b). However, despite the longevity and increasing popularity of use of propolis generally to treat many diseases, no systematic study has been reported in the use of propolis in humans with diabetic foot ulcers. The principal aim of the current work in diabetic foot ulcers was to determine if topically applied propolis on a recurrent basis is well tolerated and if it demonstrates promise as a wound healing agent. The potential benefit of propolis treatment in addition to antibiotic therapy was also investigated.

2. Research design and methods

This study was a prospective, externally (historic) controlled design. Patients with type 1 or type 2 diabetes attending the High Risk Foot Service (HRFS) at Royal Prince Alfred Hospital Sydney across the 2011 calendar year, and who fulfilled the study inclusion criteria, as described below, were invited to take part. The HRFS is a well-established multidisciplinary foot care service where we have previously reported outcome data related to wound biomarkers (Liu et al., 2009) and bacterial counts (Xu et al., 2007) in foot ulcer healing. In this study, $n = 24$ serial patients were recruited, while three other patients declined to take part. The protocol was approved by the ethics committee of Sydney South West Area Health Service, NSW, Australia, and informed consent was obtained from each enrolling patient.

A foot ulcer of 4 weeks' duration or more was deemed to be classified as a chronic ulcer, as adopted by the American Diabetes Association, (1999) and included in this study. For study inclusion, patients with a chronic foot ulcer needed to be at or above 18 years of age, with diabetes mellitus and able to give informed consent. All ulcers included in the study were classified by the established University of Texas grade and staging system, which predicts ulcer healing outcomes (American Diabetes Association, 1999). Ulcers were also described as 'neuropathic', 'neuro-ischaemic' or 'post-operative/pressure related', to help distinguish the type of ulcer category, which as described by others typically have different healing outcomes (Oyibo et al., 2001). Study exclusion criteria were: (i) patients with severe peripheral arterial disease (PAD) defined as ischemic pain at rest and/or ankle-brachial pressure index (ABPI) at or below 0.7, as these wounds were deemed unlikely to heal in the absence of revascularization (Stadelmann & Digenis, 1998); and/or (ii) foot ulcers with attendant severe infection, defined as those deemed by High Risk Foot Service medical staff to require intravenous antibiotics and/or hospital admission.

Propolis in aqueous liquid form sourced in Australia, (Honey Spring Variety, batch number 7232, Vastrade, Lidcombe NSW), was administered to cover the entire ulcer each time the patient attended from week 0 in the clinic for 6 weeks, or until the ulcer healed, whichever occurred first. A thin and even coating of propolis was painted onto the entire wound surface with a sterile cotton bud. The study personnel (FH) who applied the propolis was not involved in ongoing patient care, nor in determining ulcer area. The propolis was applied at the conclusion of each scheduled treatment, just prior to application of dressings, to minimize any potential bias from any change in routine care. The average time between visits was 10.5 days, with most individuals being seen weekly or fortnightly for standard care as is usual practice in the HRFS. This time frame of application was timed to be in keeping with the usual attendance times of patients to the Clinic, including the historic controls used in this study.

Each subject was followed up for a further 6 weeks after propolis treatment ceased, or until their wound healed, whichever occurred first. At each visit wound area was measured using acetate tracing and was scanned onto a PC all as previously described (Liu et al., 2009; Xu et al., 2007), and measured using Bersoft Image Measurement (BIM) analysis (Bersoft.com). Comparison with previous tracings enabled wound closure to be determined as a percentage of original wound area, per unit time.

In addition, on each occasion where an adequate volume of sample could be obtained ($n = 25$ μl), following ulcer debridement but prior to the application of propolis, 2×25 μl samples of wound fluid were obtained from study subjects using a calibrated sterile paperpoint tip, (Meta Biomed Co., Elmhurst, NY). The samples were mixed with 100 μl PBS and stored frozen at −80 °C for subsequent protein analysis. Samples used for bacterial count analysis were placed at 4 °C and the samples were then distributed within 2 hours onto blood agar plates.

To quantitate bacterial load, 10 μl of the post-debridement wound fluid supernatant was serially diluted (10^{-2} to 10^{-7}), then streaked onto blood agar plates, and incubated aerobically in 5% CO_2 at 37 °C for 24 hours. The number of colony-forming units (CFUs) on each plate was counted. Bacterial species were identified by standard microbiological techniques, including Gram stain, automated identification of isolates (Vitek2, Biomérieux) and susceptibility testing of *Staphylococcus aureus* isolates to determine methicillin-resistance. Previous studies in our laboratory have verified the reproducibility of sampling in a post-debridement wound fluid sample by this method (Xu et al., 2007). For matrix metalloproteinase determination, frozen wound fluids were thawed and analyzed for wound fluid MMP-2 and MMP-9, by zymography using established techniques (Liu et al., 2009).

As an external control, ulcer healing results were compared with the cohort of recently treated historical controls ($n = 84$) derived from high risk foot clinic patients with ulcers, subject to the same inclusion/exclusion criteria and receiving ongoing care in the same HRFS. Notably, the standard of care provided in the HRFS had not changed from the historic controls and the propolis treated series. All study subjects who would have qualified for the propolis study and who were treated in the same HRFS but were treated in recent years prior to study recruitment for the propolis active treatment, were included as controls. This historic control group of $n = 84$, was derived from across 2008 to 2010 calendar years. During those years the standardized approach to treating DFU in the clinic was the same as in 2011–2012 inclusive, and attendant senior medical, nursing and allied health staff were similar and in continuity across the 5 years. Treatment consisted of careful assessment of ulcer precipitating and predisposing factors with ulcer classification, followed by multidisciplinary management including pressure off-loading, debridement and dressings, and treatment of clinical infection, all as previously described in our Service (Xu et al., 2007) and following international

Table 1
Demographic and treated ulcer details of the propolis treated and historic control populations.

		Control	Propolis
Patient number (n)		84	24
Males (%)		76.2	84.0
Age (years)		63.1 ± 13.7	58.1 ± 11.2
Type 2 diabetes (%)		86.9	77.3
Diabetes duration (years)		17.7 ± 16.5	18.3 ± 9.2
Smoker (%)	– Current or past	62.3	63.6
HbA1c level (%)		8.8 ± 2.0	8.2 ± 1.6
Previous amputation (%)	Yes	15.7	32.0
Previous foot ulcer (%)	Yes – any	59.6	56.0
Ulcer area (mm^2)		219 ± 326	240 ± 561
University of Texas grade (%)	1	71.3	77.3
	2 or 3	28.7	22.7
University of Texas stage (%)	A	21.3	31.8
	B	65.0	54.5
	C or D	13.7	13.7
Ulcer type (%)	Neuropathic	78.6	66.7
	Neuro-ischemic	9.5	25.0
	Post-surgical or pressure/trauma	11.9	8.3
A recurrent ulcer site (%)	Yes	21.5	13.6
Antibiotic therapy – oral	(%)	80	75

Data are expressed where shown as prevalence (%) or as mean ± SD.

standards of DFU care (Cavanagh et al., 2005). Those treated with propolis as described earlier, received topical propolis in addition to this foot ulcer care. While hyperbaric oxygen (HBO) therapy has an evidence-base to support its use in DFU care (Liu, Li, Yang, Boden, & Yang, 2013) especially in recalcitrant non-healing DFU, it is not our usual practice nor that of others (Cavanagh et al., 2005), to use such therapy routinely, and no DFU in the propolis or control group was treated with HBO therapy in these series.

Comparison of the control subjects with propolis treated subjects showed that in general the two groups were similar in demographic and ulcer characteristics (Table 1). Notably, the diabetes duration and gender distribution were not different, nor were the HbA1c level documented at the time of clinic therapy, or a history of past foot ulceration. The average patient age and number of subjects in the propolis group who had type 2 diabetes were lower than the control group data, but neither reached statistical significance. The ulcer area, mainly forefoot site, University of Texas staging and grading of ulcers (Oyibo et al., 2001) were each similar among the propolis and control groups (Table 1). However, the propolis group had a ~ two fold higher rate of historic amputation (usually a digit), and a more than two-fold greater prevalence of neuroischemic based ulceration, compared with the control group, while the control group had a higher rate of recurrent ulceration (Table 1). Overall, the demographic and ulcer data were similar between groups and they are consistent with those reported by others in recent international literature where DFUs were managed at a high risk foot clinic (Oyibo et al., 2001; Prompers et al., 2008).

In considering treatments provided, for oral antibiotic therapy (Table 1), there was little variation between the control and propolis treated groups, with 80 and 75% respectively being on oral antibiotic treatment at study recruitment. Anti-*S. aureus* antibiotic therapy predominated in each group with $n = 66$ of 84, and 18 of 24 respectively in each group receiving treatment. Patients in both groups received routine, appropriate optimized offloading for their ulcer type (offloading types included CAM walkers, post operative shoes, felt padding, medical grade footwear and total contact orthoses) (Liu et al., 2009). The types of dressings used in the 2 groups were also similar as per the HRFS approach to wound care, with almost all patients in each group receiving foam dressings (mainly Biatain™) and occasionally, anti-microbial dressings (mainly Iodosorb™) (Liu et al., 2009).

3. Statistical analysis

All data are expressed as mean ± standard error of the mean, and are articulated in a linear scale. The presentation of MMP data is transformed logarithmically in order to realize a graphically-accessible dependent variable in this data set. T-tests (two tailed) and chi-squared test were used for demographics in Table 1, and Mann–Whitney U-tests were undertaken for ulcer healing rate, bacterial count between group comparisons, and MMP changes between groups, as data for each were not normally distributed. The chi-squared test was also used in comparing % of ulcers healed per time point.

4. Results

4.1. Ulcer healing

The main ulcer end-points examined across propolis and control groups were the rate of healing and the percentage of ulcers progressively fully healed in the study period. At weeks 1 and 3 propolis treated ulcers had a significantly greater healing rate compared with the control group (Fig. 1A). Ulcer area was reduced

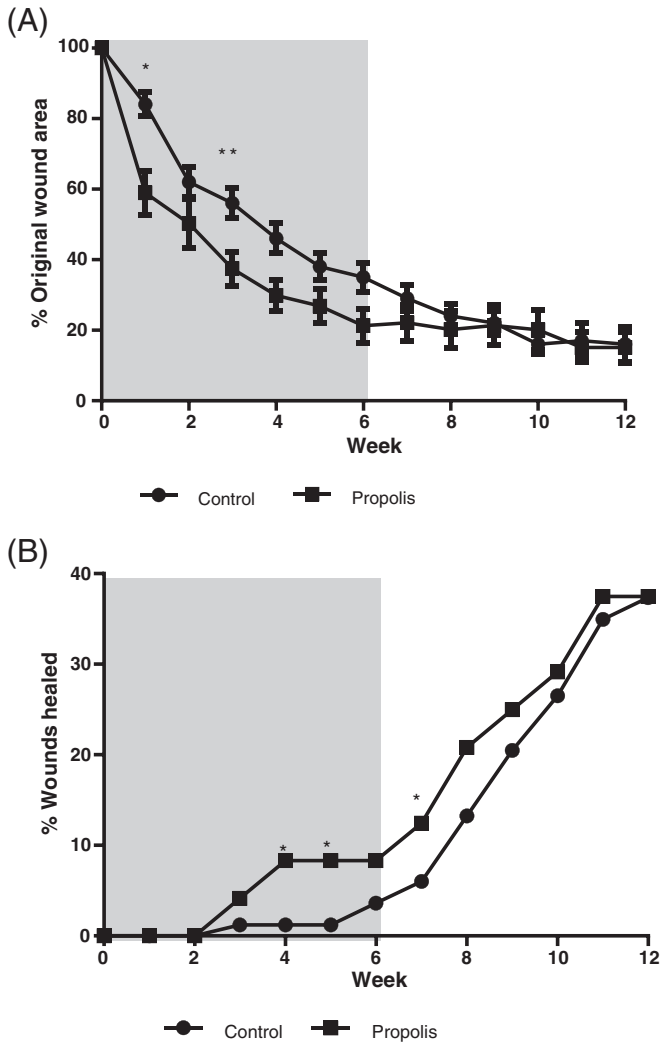


Fig. 1. Overall wound healing outcomes. (A): Percentage wound area compared to original size per week in propolis vs. historic control populations. Results are mean ± SEM. Ulcer area was reduced in the propolis group compared with the control group at week 1 (*$P < 0.001$), and at week 3 (**$P < 0.05$), respectively by Mann–Whitney U-test. (B): Percentage of patients whose ulcers had healed per week in propolis vs. historic control populations. Results are mean ± SEM. *$P < 0.05$ by chi-squared test. The shaded areas in each graph reflect the propolis treatment period in the propolis treated group.

by a mean 41% in the propolis group compared with 16% in the control group at week 1 ($P < 0.001$), and by 63 vs. 44% at week 3, respectively ($P < 0.05$), each by Mann–Whitney U-test. Subsequently, after propolis treatment was completed, the residual wound area and ulcer healing rates converged between the two groups (Fig. 1A). The percentage of patients whose ulcers were fully healed at weeks 4, 5 and 7 was higher in the propolis treated group than the controls, Fig. 1B. At weeks 4 and 5, 10 vs. 2% ($P < 0.001$), then by week 7, 13 vs. 5% ($P < 0.05$) of propolis treated vs. control ulcers had fully healed, respectively. As per the ulcer healing rate, after propolis therapy had been completed, the curves for the percentage of ulcers that had healed converged for the propolis and control groups (Fig. 1B).

In a pre-planned exploratory analysis, study subjects were sub-divided into two groups, those that were treated with antibiotic therapy, and those who were not. Ulcers of patients who were receiving systemic antibiotic therapy as well as topical propolis ($n = 16$) showed improved healing rate at weeks 1, 3 and 4 compared with ulcers that received systemic antibiotics only ($n = 66$), with $^{*}P < 0.001$ at week 1, and $^{**}P < 0.05$ at weeks 3 and 4, by Mann Whitney U-test, Fig. 2A. No such differences were seen when the non-antibiotic treated control group ($n = 18$) and propolis treated ulcers without antibiotics ($n = 8$), were compared (not shown). Control group ulcers receiving no antibiotics showed accelerated healing at weeks 2 and 3 compared with control group antibiotic treated wounds (each $^{**}P < 0.05$ by Students t-test, Fig. 2B). In contrast, no differences were observed within the propolis treated ulcers for those receiving antibiotic or non-antibiotic therapy, as per Fig. 2C. As addressed in the subsequent discussion, only clinically infected ulcers in the Propolis study were

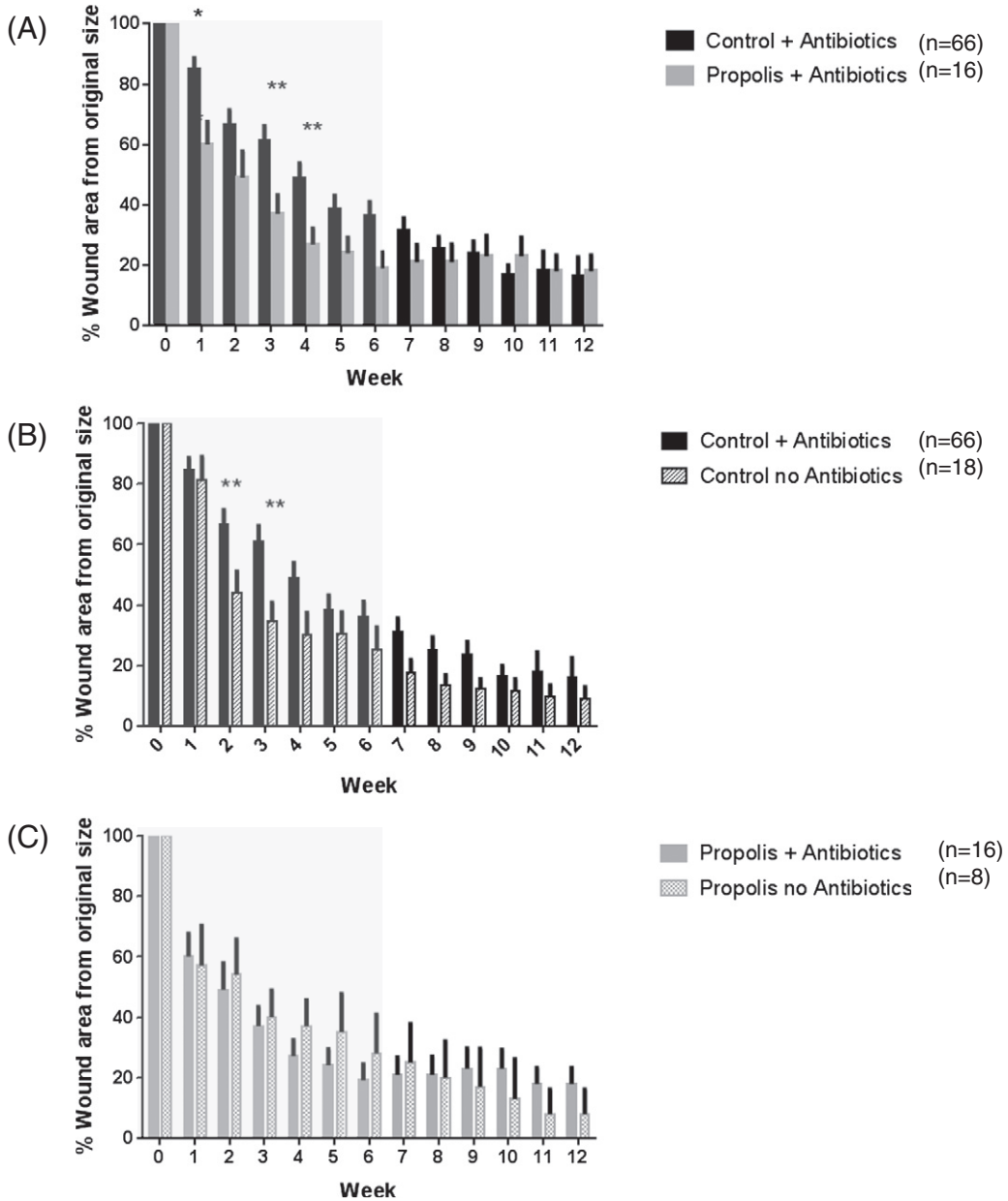


Fig. 2. Wound healing outcomes based on concurrent antibiotic therapy. (A): Propolis vs. historic control populations each of whom were receiving concurrent antibiotic therapy. (B): Comparisons in the control population who were receiving antibiotic concurrent oral antibiotic therapy compared with the controls who were not. (C): Comparisons in the propolis treated population who were receiving antibiotic concurrent oral antibiotic therapy compared with the propolis treated who were not. In each case, results are mean ±SEM. $^{*}P < 0.001$ and $^{**}P < 0.05$ vs. respective comparator during the same treatment week, by Mann–Whitney U-test.

treated with antibiotic therapy. These data, although limited by sample size, suggests that propolis may particularly help healing of antibiotic treated, clinically infected ulcers.

4.2. Bacterial load and profile

In subgroup analysis, bacterial load was compared early after treatment between visit 1 and 2 in subjects treated with propolis. After treatment with propolis, the number of colony forming units (CFU's) counted was reduced per week by 17%, from 118 to 88 CFU's in the propolis treated group. While this change was not statistically significant, possibly related to small sample size and inter-individual variation in data, it represents a 26% reduction in bacterial burden in the wound across 10 days (Fig. 3). In general, across visits 1 and 2, all bacterial species reported appear to have reduced in CFU counts (Fig. 3). This rate of change of CFU data was compared with CFU counts in ulcer fluid obtained from 32 patients with neuropathic ulcers from our previous study published in 2007 (Xu et al., 2007). This latter cohort comprised similar patients who were referred to the same HRFS as the propolis study and received similar care. The CFU count showed a significant change across 10 days of −26% in the propolis treated group, compared with a reduction of less than −1% in the published control group ($P < 0.001$, by Mann Whitney U-test).

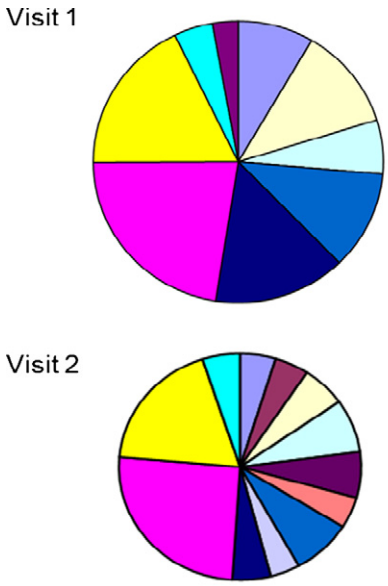
4.3. MMP-2 and MMP-9 changes in wound fluid

Wound fluid samples from propolis treated study participants were analyzed by gelatin zymography. By this techniques and shown in the representative image (Fig. 4A) it is possible to determine the type of MMP and its functional status. The smaller form of MMP is the activated MMP and in this work referred to as 'active' MMP while the form with higher molecular weight is biologically latent and is termed the pro MMP. Also shown in Fig. 4A are representative results from a propolis treated ulcer, where changes in active MMP-9 are observed from visit 1 to visit 2, each run in duplicate. The change of active MMP-2 and -9 levels were calculated as a percentage change per 10 days. Rate of change in wound area (%) was also calculated over the same period. The MMP data were normalized by log transformation and expressed as % change between visits 1 and 2, and visits 2 and 3. As shown in Fig. 4, from visits 1 to 2, active MMP-9 fell in the propolis treated ulcers (Fig. 4B), and was unchanged from visits 2 to 3 (Fig. 4C). Active MMP-2 did not change across the intervals studied (Fig. 4 and C), and the amounts of pro-MMP-9 or pro-MMP-2 were also not altered (not shown).

These data were compared with a subgroup of patients ($n = 39$) who were seen at the same time-points as the Propolis treated cohort, that were a subgroup of the historic controls referred to for ulcer healing in the current report (Suk et al., 2009). For this analysis archived results for wound fluid MMP levels at the initial and subsequent visits were assessed as a rate of change between visits as described. The active MMP-9 levels decreased at a significantly faster rate in the propolis treated group when compared with the control group in the first time period (visits 1 and 2), $P < 0.05$ by Mann–Whitney U-test (% change per 10 days $\log_{10}$ −0.68 for propolis vs. 2.64 for controls), Fig. 4B. This pattern was maintained in the second time period (visits 2 and 3), $P < 0.005$ although the magnitude of the change was not as great (% change/10 days, 0.05 for propolis vs. 0.64 in controls). Across each time period, no differences in rate of change of active MMP-2 were observed between propolis and the controls, as shown in Fig. 4B and C.

4.4. Tolerability and safety

There were no adverse effects observed in any of the propolis treated study subjects including absence of cutaneous allergy. In personal communication, all regular staff at the foot Clinic indicated that the administration of propolis to a foot ulcer added to normal care had no adverse impact on the day-to-day activities of the Clinic. The staff stated that they would consider using propolis routinely as a topical wound therapy after it had been formally tested in a randomized controlled trial in diabetic foot ulcers.



	Visit 1 (%)	Visit 2 (%)
Alpha-haemolytic streptococcus sp	9	5
Bacillus sp	0	5
Corynebacterium sp	12	6
E. cloacae	6	8
E. coli	0	6
E. faecium	0	4
Enterococcus sp	11	8
Neisseria sp	0	4
P. aeruginosa	15	5
S. aureus	22	25
S. epidermidis	18	19
S. marcescens	4	5
Proteus sp	3	0

Fig. 3. Effect of propolis on post-debridement wound fluid viable bacteria. The pie charts show types of bacteria identified by count of colony forming units at (A) visit 1 and (B) visit 2. The relative area of each pie chart in (A) and (B) reflects relative CFU counts. The differing bacteria cultured on blood agar after 24 hours are shown, with percentages of bacteria at each respective visit, totaling 100% for all bacteria combined. The average time difference between visit 1 and visit 2 was 10.5 days and the range, 1–2 weeks.

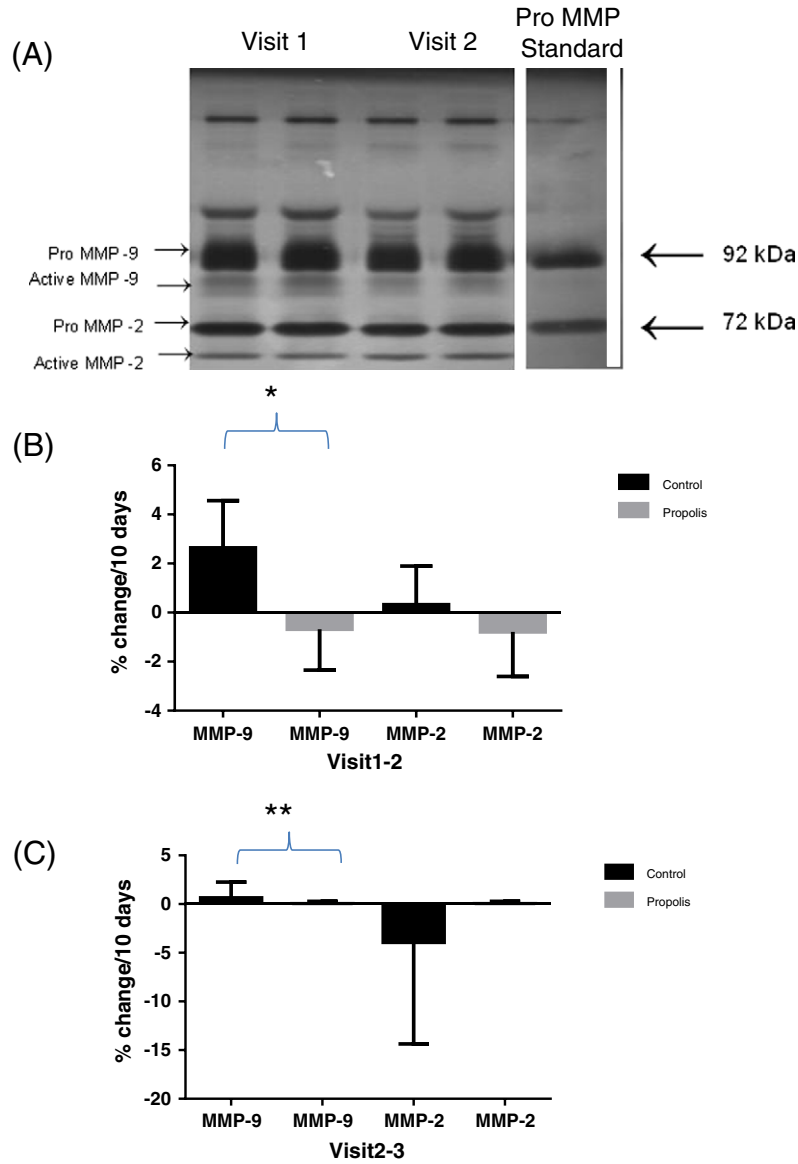


Fig. 4. MMP-2 and MMP-9 changes in post-debridement wound fluid. (A): Representative zymography image showing pro- and active-MMP-2 and MMP-9 with standards, for a patient ulcer treated with propolis, with samples run in duplicate for visits 1 and 2; active-MMP-9 signal is seen to fall from visit 1 to visit 2. Active MMP-9 and active MMP-2 changes from (B) visit 1 to visit 2 and (C) visit 2 to 3, each per 10 days are shown, in control and propolis treated wounds across the visits indicated. Data are mean ± SEM. The *y*-axis data are log transformed in each case. At visits 1–2 and 2–3 active MMP-9 change was significantly different in the propolis treated group compared with controls; $^{*}P < 0.05$ and $^{**}P < 0.005$ respectively compared with controls, by Mann Whitney U-test. No such differences were seen in MMP-2 levels.

5. Discussion

The known anti-inflammatory and anti-bacterial properties of propolis combined with positive preclinical data in diabetic ulcers (McLennan et al., 2008) make it a natural target for a human wound healing study in diabetes. Previous studies of propolis have indicated that it has low allergenicity to humans (Rajpara et al., 2009), low financial cost and shows wound healing acceleration in a diabetic rat model (McLennan et al., 2008). The current single site, prospective, externally controlled study demonstrates that propolis is generally well tolerated by patients, it is straightforward to apply by staff, and it has findings of efficacy in ulcer healing. Both initial ulcer healing rate and healing completeness, as well as wound microenvironment measures of active MMP-9 and bacterial count were improved by propolis. To our knowledge, while individual case reports have implicated propolis as having a role in diabetic ulcers (Lotfy, Badra, Burham, & Alenzi, 2006), this is the first report on outcome of a series of systematically treated diabetic foot ulcers.

It is notable that the effect of proplis was most clearly seen within some weeks of its first topical application. Advantage of propolis on ulcer healing rate was seen at weeks 1 and 3, and on overall healing on week 4. These quite rapid effects are consistent with the known potent anti-inflammatory effects of propolis, and its efficacy in the rodent model of diabetic ulcer healing. The current pilot study was designed to optimally detect these early differences. In contrast, the ulcer healing rates began to rapidly converge after propolis treatment had completed (after week 6), as did the overall ulcer healing rate. These data suggest that the effect of propolis is relatively transient. It is unknown whether the efficacy of propolis observed during its application would have persisted if the therapy was maintained.

The primary aim of this pilot study was to evaluate the effectiveness of recurrent administration of topical propolis in a patient series. An indication of effect size, feasibility, acceptability and adverse events, of topical propolis in chronic diabetic foot ulcers was also sought. Propolis application with each HRFS clinic visit demonstrated no adverse effects

on patients or their ulcers, and its use was well accepted by regular clinic staff. In using the data acquired, predictions of an appropriate sample size and related study design can be made for a future full scale randomized controlled study of proplis in diabetic foot ulcers. The current pilot feasibility study was not powered to determine if propolis may be effective in foot ulcer healing. It did however show that propolis was safe, well tolerated and easy to use and that propolis may have efficacy in diabetic foot ulcer healing. Sheehan and colleagues reported that achieving 50% in wound healing area by week 4 is a robust predictor of long term healing prognosis (Sheehan, Jones, Giurini, Caselli, & Veves, 2006). That is, if a diabetic foot ulcer wound is not 50% healed at week 4 then there is only a 9% chance it will be healed completely within 3 months. Power calculations based on this pilot study show that in order for propolis treated wounds to show a 40% improvement in healing compared with wounds receiving standard care at week 4 with confidence level of 0.95, that is for the propolis treated group to be 30% of original size and control wounds to be 50% of original size, also with variance as in this study, for 80% power, $n = 103$ subjects would need to be recruited in each arm of a randomized controlled study of propolis vs. control.

Significant improvement in healing rate was seen in the overall cohorts and when the antibiotic treated control and propolis subgroups were compared. No such differences were seen among the two non-antibiotic treated groups. It is also possible that the effects of propolis are predominant in clinically infected ulcers as they required antibiotic therapy. It should be noted that all of the propolis treated ulcers deemed clinically infected, as per Texan staging at 67% ($n = 16$ of 24), were each treated with antibiotics, and that our Service have a low threshold for prescribing and maintaining ongoing antibiotic therapy until ulcer healing. No clinically uninfected ulcers in the propolis study were treated with antibiotics. This subgroup finding is consistent with the known anti-bacterial effects of propolis (Astani et al., 2013). In addition, in the control group, healing rates were greater at weeks 2 and 3 in the wounds that did not receive antibiotic therapy. It is logical that these wounds in the control group healed more rapidly because they lacked the complicating factor of infection. This situation was not seen in the propolis treated infected wounds. The healing rate between the antibiotic treated and non-antibiotic treated propolis treated wounds was similar. This finding further supports the concept that the anti-bacterial properties of propolis have prevented the delay in healing that would otherwise occur in the infected wounds. They support the recent finding that propolis potentiates the potency of most antibiotics, especially those active against *S. aureus* (Wojtyczka et al., 2013). Future studies should examine these preliminary data, in a larger, randomized controlled trial design.

Propolis had demonstrable antibacterial effect in this study. The bacterial load (CFU) in propolis treated ulcers decreased by 26% in diabetic wound fluid over 10 days compared with <1% in a subgroup of published controls. Studies by our laboratory found that increased bacterial count predicts poor wound healing in neuropathic ulceration, the predominant ulcer type in this study (Xu et al., 2007). Multiple studies have previously elucidated that propolis is able to reduce bacteria in wounds (Erkmen & Ozcan, 2008; Kosalec, Pepeljnjak, Bakmaz, & Vladimir-Knezevic, 2005; Mihai et al., 2012), mainly with bacteriostatic activity (Drago et al., 2000). This is particularly the case for *Pseudomonas aeruginosa* (Farnesi, Aquino-Ferreira, De Jong, Bastos, & Soares, 2009; Pepeljnjak & Kosalec, 2004). Our pilot study was too small to examine effects on specific bacterial species. Studies have explored the mode of action of propolis and found that ethanol extract of propolis (EEP) is able to regulate gene expression of bacteria; EEP is able to significantly affect the LasA and LasB (staphylolytic endopeptidases secreted by *P. aeruginosa*) gene expression and protease activities (Morales et al., 2011). These changes in the bacterial protease activity were observed with no significant effects on survival of the organism. This finding suggests the need for further investigations to be made on the effect of EEP on the maturation and differentiation of bacterial biofilms, including *in vivo*.

The protease MMP-9 in wound fluid has importance as a marker, and a potential mediator, of foot ulcer healing in diabetes. High levels of active MMP-9 have been shown to correlate with poor wound healing and to predict future non-healing (Ladwig et al., 2002; Liu et al., 2009). Reductions in the expression of MMP-9 preclinically, correlate with increased wound healing and percentage of collagen in wounds in a diabetic rat model (Aparecida Da Silva et al., 2013) and caffeic acid phenyl ester (CAPE) a component of propolis has potent ability to inhibit the protein concentration of pro-inflammatory proteinase, matrix metalloproteinase-9 *in vitro* (MMP-9) (Ladwig et al., 2002). In the current study propolis treated wounds showed significantly greater reductions in the active MMP-9 compared with an untreated control subgroup. How propolis causes this change is uncertain and whether some of the actions are via effects on regulators of MMP activities such as the tissue inhibitors of metalloproteinases are possible mechanisms.

Propolis contains more than 180 separate compounds and while its ingredients differ based on plants in different geographic regions accessed by propolis making bees, the main active components are thought to be present in all forms of propolis (Wagh, 2013a). The current study used a readily available commercial source of propolis. Propolis' anti-inflammatory effects are largely attributable to CAPE (Ladwig et al., 2002; Nagaoka et al., 2003; Natarajan, Singh, Burke, Grunberger, & Aggarwal, 1996; Russo et al., 2002) and its anti-bacterial activity is thought to be mainly due to the phenolic acid fraction (Wojtyczka et al., 2013). Robust evidence exists to support the anti-microbial properties of propolis. It has an inhibitory concentration that is 400 times greater than tetracycline's against *Escherichia coli* and more than 50 times higher against *S. aureus* and *Bacillus subtilis* (Bonvehi & Coll, 1994). Propolis has also been shown to inhibit the proliferation of fungal elements such as *Candida albicans* (Metzner, Schneidewind, & Friedrich, 1977) and viruses (Gekker et al., 2005).

Despite its potential as an agent to improve outcomes in many disease states, published blinded randomized controlled trials of topical cutaneous propolis therapy are notably lacking. Other topical therapies in DFU healing also hold promise, such as uncontrolled pilot studies of certain impregnated protease inhibitor ulcer dressings (Richard et al., 2012) and anti-bacterial iodine based preparations (Schwartz et al., 2013). Furthermore, treatments such as topical phenytoin impregnated dressings have shown some efficacy in small, randomized studies in DFU (Shaw, Hughes, Lagan, & Bell, 2007), although subsequent high quality randomized controlled trial has not shown benefit (Shaw et al., 2011). While a clinical trial with blinding of a propolis treatment group would be difficult due to its characteristic color and odor, the current work can be used as the justification for a larger scale, multicentre, likely open label, randomized controlled trial to determine if propolis, in combination with wound care to an international standard (Cavanagh et al., 2005) undertaken in a multidisciplinary high risk foot service setting (Xu et al., 2007), significantly improves the healing of diabetic foot ulcers compared with standardized wound care alone. This current pilot demonstrates that recurrent topical propolis therapy is well tolerated and feasible and that randomized controlled trial assessment of topical propolis appears to be warranted in diabetic foot ulcers.

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