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Tropical ulcer plant treatments used by Papua New Guinea's Apsokok nomads

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Abstract

Ethnopharmacological relevance

The tropical ulcer is a debilitating bacterial infection that is common in Papua New Guinea. Deploying healthcare infrastructure to remote and inaccessible rainforest locations is not practical, therefore local plants may be the best treatment option. Here we present an ethnobotanical survey of the tropical ulcer plant medicines used by the semi-nomadic Apsokok who roam the remote central mountains of Papua New Guinea's West New Britain Province. *In vitro* biological activity in assays relevant to tropical ulcer wound healing is also presented.

Materials and methods

Focus groups and semi-structured interviews were used to acquire information on the uses of plants, vouchers of which were identified by comparison with authentic herbarium specimens. Antibacterial disc diffusion assays with *Staphylococcus aureus* and *Fusobacterium ulcerans*, MMP-9 enzyme inhibition and dermal fibroblast stimulation assays were carried out on plant saps and aqueous extracts of plant material. LC-MS was used to identify known plant metabolites.

Results

The ethnobotanical survey identified sixteen species that were used to treat tropical ulcers, all of which were applied topically. A subset of twelve species were investigated further *in vitro*. Four species produced zones of inhibition with *S. aureus*, all 12 species provided low level inhibition of MMP-9 and 8 species stimulated dermal fibroblast proliferation, although cytotoxicity occurred at higher concentrations. The extract of *Homalium foetidum* Benth. inhibited *S. aureus* and MMP-9 while at lower sub-cytotoxic concentrations stimulated fibroblast proliferation. Trans-3-O-p-coumaroylquinic acid cis-3-O-p-coumaroylquinic acid were detected in the aqueous extract of *H. foetidum*.

Conclusions

Topical application of plant saps to wounds results in very high localised concentrations of plant metabolites which is likely to result in inhibition of MMP proteases. *H. foetidum* is a candidate plant for tropical ulcer treatment in remote areas.

1. Introduction

The tropical ulcer is an extremely painful and debilitating bacterial infection (Lupi et al., 2006). It commonly affects children and adolescents and presents as a rapidly growing ulcer of the lower leg (Adriaans and Drasar, 1987). Treatment options include skin grafting, antibiotics such as metronidazole, and topical antiseptics such as gentian violet (Singal, 2015). Evidence suggests that treating tropical ulcers may place a considerable burden on government aid posts in Papua New Guinea with up to a third of their time and half their budgets spent treating the condition (Morris et al., 1989). For remote populations in Papua New Guinea, treatment options are extremely limited. It is not practical for the government to deploy basic healthcare infrastructure across remote areas which are not even accessible by light aircraft. There is therefore a strong rationale for early treatment of small bacterial skin infections with locally available antibacterial plants rather than late treatment of chronic tropical ulcers in a hard to reach clinic. Furthermore, in Papua New Guinea, for populations that have access to healthcare, antibiotics are widely available and are even applied topically to tropical ulcers, a practice which would be expected to contribute to the development of antibiotic resistance. Using antibacterial plants as first line topical antiseptics, may help reduce this risk.

Tropical ulcers start as infected scratches or insect bites but may become several centimetres in diameter within a couple of weeks (Lupi et al., 2006). The ulcers have a well-defined undermined edge and a characteristic foul smelling slough overlaying a soft and easily bleeding base of inflamed tissue (Adriaans and Drasar, 1987; Falkler et al., 1989). The aetiology of the ulcer remains unclear; attempts to isolate the causative pathogen(s) have yielded *Treponema spp.*, fusiform bacteria such as *Fusobacterium ulcerans*, *Staphylococcus aureus, Corynebacterium haemolyticum* and more recently *Haemophilus ducreyi* suggesting some or all of these organisms are involved in the pathogenesis (Adriaans and Shah, 1988; Bowness et al., 1984; Lupi et al., 2006; Mitja et al., 2014).

In the present study we present an ethnobotanical survey of plants used to treat tropical ulcers by a little known semi-nomadic population who roam deep into Whiteman Range in the mountainous interior of West New Britain Province (Fig. 1). They refer to themselves as Apsokok and have apparently broken away from larger settled Apsokok communities that live in established villages on either side of the Whiteman Range. This population of less than a fifty people, consists of a small number of family groups each living in temporary open sided shelters in widely dispersed forest clearings 100m in diameter. The nomadic Apsokok are able to recall a time before the Second World War when they lived in forest on the South Side of the Whiteman Range; startled by the appearance of wartime aircraft they moved deeper into the interior of the island. In the 1970s most family groups settled at the village of Ishmin on the Kulu River enabling them to find employment in the surrounding oil palm plantations. The remaining family groups who have opted to continue a semi-

nomadic lifestyle inland, periodically visit their relatives in Ishmin to acquire essential supplies such as salt, and metal implements. For this small, hard to access population, it would be useful to identify which plant species could be used in the place of conventional first line treatments.
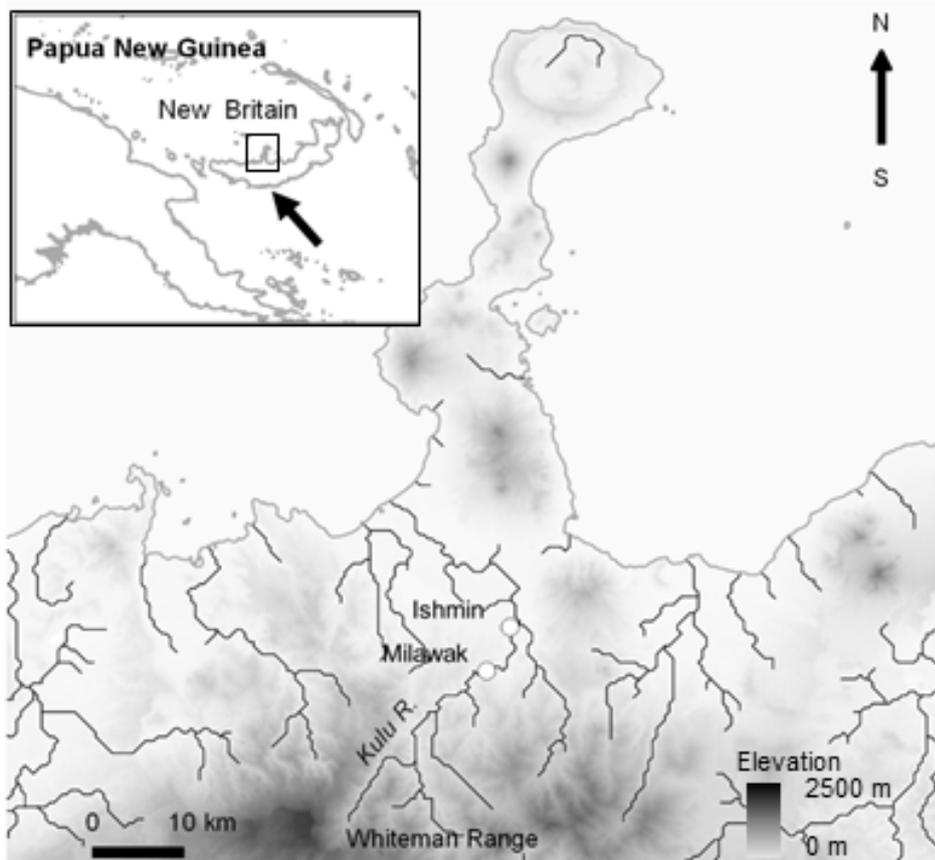
Fig. 1. Location of Ishmin village and the temporary settlement of Milawak in West New Britain Province, Papua New Guinea. The ethnobotanical survey was carried out in small rainforest clearings within a 2 km radius of Milawak. Rainforest clearings can be observed on satellite imagery along the upper branches of the Kulu River and these are consistent with geographical descriptions of temporary Apsokhok camps given by informants.

2. Materials and Methods

2.1 Ethnobotanical data collection

Consent for this study was obtained from local participants and the government of Papua New Guinea prior to commencement. In May 2014, informal interviews were carried out with a key informant from one of the semi-nomadic families in a forest clearing at S 5° 39.69 E 150° 0.14 and this information was used to provide a provisional list of medicinal plants. In March 2016 more extensive interviews were carried out with several informants living downriver in the small settlement of

Milawak S 5° 39.37 E 150° 0.89 and two other small clearings nearby. This allowed the names of tropical ulcer plant medicines to be expanded from information recorded in 2014 and in most cases corroborated through separate interviews with informants in these new locations. All interviews were conducted in Neo-Melanesian (Tok Pisin) language. As described previously, care was taken not to overly rely on data from any single informant (Etkin, 1993).

2.2 Collection and identification of plant material

Voucher specimens were collected from forest surrounding the temporary hamlet of Milawak in the presence of key informants and preserved in 70% methanol before pressing and drying. Identification of vouchers was carried out by comparison with authentic herbarium specimens at the Royal Botanic Gardens, Kew, UK and the National Herbarium, Lae, PNG where vouchers are lodged. In addition to voucher specimens, plant material for laboratory tests was collected separately into dry silica gel; plant saps were collected into PCR tubes, before storage at -20 °C.

2.3 Extraction of plant material

Plant saps were centrifuged at $13400 \times g$ before sterile filtration with a 0.02 µm PTFE syringe filter. Silica dried plant material was ground to a powder in a mortar and pestle, 50 mg of which was extracted with 0.4 ml water for several hours before centrifuging twice at $13400 \times g$ followed by sterile filtration with a 0.02 µm PTFE syringe filter.

2.4 Antibacterial disc diffusion assays

Fusobacterium ulcerans (NCTC 12112) was cultured in an anaerobic cabinet at 37 °C in anaerobic broth (Lab M) and adjusted to OD_{600} 0.08-0.13 (0.5 McFarland). Nutrient agar plates (Lab M) were inoculated with 200µl culture and fully dried before adding four sterile discs (Whatman Grade AA, 6mm diameter) containing 15µl of sterile filtered plant extract. A positive control of 15µl 10% povidone iodine (Vetasept) was also included. Plates were incubated for three days in an anaerobic cabinet before measuring zones of inhibition in four locations to obtain an average diameter.

Staphylococcus aureus (NCTC 6571) was cultured in Mueller Hinton broth (Oxoid) and adjusted to OD_{600} 0.08-0.13 (0.5 McFarland). Mueller Hinton agar plates were inoculated with 200µl culture before adding paper discs containing test substances as above. Plates were incubated aerobically for three days at 37 °C before measuring zones of inhibition.

2.5 Matrix metalloproteinase inhibition assay

The MMP-9 colorimetric drug discovery kit (Enzo Life Sciences) was adapted to a 384 well plate format using a total working volume of 25 µl per well. The kit includes recombinant MMP-9 and a chromogenic thiopeptide which when cleaved reacts with Ellman's reagent resulting in increased

absorbance at 412nm. Control experiments were carried out in which the extracts were incubated with the assay reagents without enzyme to check for non-enzyme induced increases in absorbance at 412nm. Secondly, a concentration of 0.0625mM 2-Mercaptoethanol was used to artificially induce a colour change equal to that of the enzyme, extract concentrations were lowered to the point where they did not interfere with this.

2.6 Fibroblast stimulation assays

Normal adult human dermal fibroblasts (Lonza) were cultured in BulletKit medium (Lonza) and seeded in 96 well plates at 5000 cells per well before replacing the culture medium with basal medium lacking foetal bovine serum. Serial dilutions of aqueous extracts were then added along with an aqueous no extract control. Separate controls for background absorbance, (medium with no cells) and full supplementation (50 µl complete medium) were also included. The plate was then incubated at 37 °C for 48 hours before replacing the medium with basal medium and adding CellTiter reagent (Promega). Absorbance at 490nm was then read in a Tecan Infinite M200 plate reader.

2.8 Chemical analysis instrumentation

LC-MS/MS analysis was carried out with a Thermo Scientific 'Accela' LC-system (autosampler, pump and photodiode array detector) coupled to a Thermo Scientific 'LTQ-Orbitrap XL' hybrid linear ion trap-orbitrap mass analyser fitted with an 'Ion-Max' electrospray ionisation (ESI) source. Samples (5 µl) were injected onto a RP C18 column (Phenomenex Luna C18(2), 150 × 3 mm i.d., 3 µm particle size) and eluted at 0.4 mL min-1 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 30 min). MS1 spectra at 30,000 resolution were recorded in the range m/z 125–2000 by the Orbitrap (FTMS) in positive mode. Simultaneously with the high resolution FTMS analysis, the linear ion-trap (ITMS) recorded low resolution MS1 (m/z 125–2000), MS2 and MS3 spectra in both positive and negative modes. A 4 m/z ion isolation window and relative collision energy of 35% was used for all MS2 and MS3 spectra.

3. Results

3.1 An ethnobotanical survey identifies sixteen plants used to treat tropical ulcers

Ethnobotanical fieldwork identified sixteen species that are used to treat a condition consistent with the tropical ulcer (table 1.). In each case the plant material is applied topically to the wound surface. Comparison with published data available for neighbouring communities such as the inland Kaulong and Miu language groups reveals little overlap. Only three of the sixteen species are used by either of those communities to treat tropical ulcers, these are *Codiaeum variegatum* (L.) used by the inland

Kaulong, *Homalium foetidum* Benth used by the Miu speaking community and *Merremia peltata* (L.) Merr also used by the Miu community (Prescott et al., 2015; Prescott et al., 2012).

Species and voucher number	Family	Local name	Description of use
Codiaeum variegatum (L.) Rumph. ex A.Juss. <u>135</u>	Euphorbiaceae	Prumen	The leaves are squeezed and the resulting exudate applied to tropical ulcers (1 informant 2014). Alternatively, the stem sap is applied to tropical ulcers. (5 informants 2016).
Cordyline fruticosa (L.) A.Chev. <u>136</u>	Asparagaceae	Maray	The leaves are broken and twisted and the resulting sap applied to tropical ulcers (1 informant 2014, 5 informants 2016).
Alstonia cf. *scholaris* (L.) R.Br. <u>137</u>	Apocynaceae	Rambaka	The sap is applied to tropical ulcers (1 informant 2016).
Homalium foetidum Benth. <u>138</u>	Salicaceae	Malas	The new leaves are applied to subcutaneous skin infections or to large tropical ulcers on the feet (5 informants 2016).
Faradaya splendida F.Muell. <u>139</u>	Lamiaceae	Tetequoi	The vine is cut and the sap blown out of the stem and allowed to drip onto tropical ulcers or cuts, (1 informant 2014). It also can be applied to burns (1 informant 2016).

Pangium edule Reinw. <u>140</u>	Achariaceae	Kali	The bark is scraped with a knife and the fluid in the moist wood shavings is applied to tropical ulcers, (5 informants 2016). Alternatively the sap is mixed with slaked lime and applied to tropical ulcers (2 informants 2016).
Curcuma longa L. <u>141</u>	Zingiberaceae	Suma	The rhizoid is chewed and the resulting mulch is applied to tropical ulcers (1 informant 2014). Alternatively the roots are crushed and mixed with slaked lime inducing a colour change from yellow to red, the coloured material is then applied to tropical ulcers (2 informants 2016).
Hornstedtia scottiana (F.Muell.) K.Schum. <u>142</u>	Zingiberaceae	Waveness	The leaves are burnt in a fire to produce ash which is applied to tropical ulcers, (1 informant 2014, 5 informants 2016).
Cayratia sp. <u>143</u>	Vitacaeae	Elik elamus	The vine is cut and the sap blown out of the vine and onto the surface of tropical uclers, (1 informant 2014 and 1 informant 2016).
Merremia peltata (L.) Merr. <u>144</u>	Convolvulaceae	Mabling	The stem is cut and the sap allowed to drop onto new cuts (5 informants 2016) or tropical ulcers (1 informant 2014).

*Parartocarpus sp.*145	Moraceae	Riku	The bark is cut and the sap applied to tropical ulcers (1 informant 2016).
Barringtonia sp. 146	Lecythidaceae	Mukru	The leaves are applied to a type of subcutaneous skin infection, (1 informant 2014 and 1 informant 2016).
Mangifera minor Blume 147	Anacardiaceae	Wakayo	The wood is scrapped with a knife to produce shavings of wood which are squeezed to produce a fluid which is applied to tropical ulcers, (2 informants 2016).
Flagellaria indica L. 149	Flagellariaceae	Sara	The leaves are burnt and the ash is applied to tropical ulcers, (2 informants 2016).
Allophylus cobbe (L.) Raeusch. 150	Sapindaceae	Elop	The wood is scraped with a knife and the wood shavings mixed with slaked lime and applied to tropical ulcers, alternatively they may be mixed with the ash of *Hornstedtia scottiana* and then applied to tropical ulcers, (1 informant 2016).
Artocarpus altilis (Parkinson ex F.A.Zorn) Fosberg 151	Moraceae	Pana	The leaves are burnt to produce ash which is then applied to tropical ulcers, (1 informant 2014 and 1 informant 2016).

Table 1. Medicinal plants used by the nomadic Apsokok to treat conditions whose therapeutic use closely resembles that of tropical ulcer treatment. Voucher numbers (Prescott T.A.K.) are underlined after the species name. Where possible, plant names are written phonetically as used in the local Aighon language; in a few cases this is the same as the Tok Pisin name.

3.2 Several species produced zones of inhibition with S. aureus but none with F. ulcerans.

An important feature of a putative tropical ulcer plant treatment is antibacterial activity against bacterial species known to be involved it the pathogenesis of the ulcer. We therefore tested the extracts against gram positive and gram negative species (*S. aureus* and *F. ulcerans*) that have been isolated from tropical ulcers in Papua New Guinea. Aqueous extracts were used in a disc diffusion assay as this resembles the *in vivo* situation in which moist plant material or plant sap applied to a wound may diffuse across the wound surface. None of the extracts produced zones of inhibition with *F. ulcerans*, however several species were active against *S. aureus* (Table. 2).

3.4 The extracts inhibited MMP-9 at concentrations well below those likely to occur in vivo

Although little is known about the tropical ulcer wound environment, research from chronically un-healing wounds that occur in the western world demonstrates that excessive protease activity from enzymes such as MMP-9 contribute towards stalled wound healing by degrading extracellular matrix and growth factors. (Uccioli et al., 2015; Wysocki et al., 1993). Therefore, inhibition of MMP-9 may be a beneficial characteristic of a putative tropical ulcer topical treatment. Fig. 2A shows that all of the extracts show modest levels of enzyme inhibition. The 0.01% v/v test concentration was selected as it was the highest concentration at which none of the extracts interfered with the colorimetric detection of cleaved thiopeptide. As extract concentration at the wound surface and in the wound exudate could reasonably be expected to reach 50% v/v, the results suggest *in vivo* MMP-9 inhibition could be readily achieved.

3.5 Several plant extracts stimulated proliferation of human dermal fibroblasts under low serum conditions

Another characteristic of stalled wound healing is a stress-induced premature senescence phenotype that may result from excessive inflammation and oxidative stress (Wall et al., 2008). As fibroblasts play a central role in wound repair through collagen synthesis and the production of cytokines, normal fibroblast activity is essential for normal wound healing (Tepole and Kuhl, 2013). With this in mind the plant extracts were tested for their ability to stimulate proliferation of human dermal fibroblasts. A maximum dose of 5% v/v was used to reflect the high concentrations of extract fibroblasts would experience at the wound surface. Seven of the plant extracts were able to stimulate fibroblast proliferation, however this was concentration-dependent; higher concentrations tended to result in

cytotoxicity (Fig. 2B-C). Interestingly *H. foetidum* stimulated fibroblast proliferation at 0.05% v/v, despite also exhibiting antibacterial activity; however the two activities occurred at different concentration ranges.

3.3 The aqueous extract of H. foetidum contains coumaroylquinic acids

LC-MS was used to gain an insight into the metabolites present in the *H. foetidum* aqueous extract. The known compounds trans-3-O-p-coumaroylquinic acid and cis-3-O-p-coumaroylquinic acid were detected (Fig. 3). These two isomers have the same MS2 fragmentation but different UV spectra (λmax = 311nm and 305nm, respectively). Their identifications were based on comparison of the observed UV and MS/MS spectra with Kew's in house ESI-MS library and data in literature (Tanaka et al., 2014; Plazonic et al., 2009). Interestingly the related compound 3-p-trans-coumaroyl-2-hydroxyquinic acid has been reported to have antibacterial activity against *S. aureus* (Wu et al., 2016).

ID	Species	Plant part	S. aureus	F. ulcerans
135	*Codiaeum variegatum*	Sap	Inactive	Inactive
137	*Alstonia* cf. *scholaris*	Sap	Inactive	Inactive
138	*Homalium foetidum*	Leaf extract	6.7mm	Inactive
139	*Faradaya splendida*	Sap	Inactive	Inactive
140	*Pangium edule*	Bark extract	Inactive	Inactive
141	*Curcuma longa*	Sap	Inactive	Inactive
143	*Cayratia sp.*	Stem extract	7.9mm	Inactive
144	*Merremia peltata*	Sap	Inactive	Inactive
145	*Parartocarpus sp.*	Sap	Inactive	Inactive
146	*Barringtonia sp.*	Leaf extract	7.4mm	Inactive
147	*Mangifera minor*	Sap	Inactive	Inactive
150	*Allophylus cobbe*	Bark extract	7.5mm	Inactive
-	Povidone iodine	-	12.2mm	18.8mm

Table 2. Zones of inhibition (diameter) of extracts tested with *S. aureus* and *F. ulcerans* in a disc diffusion assay. Each 6mm paper disc was treated with 15 μl aqueous plant extract or 15μl 10% povidone iodine before applying to agar plates seeded with *S. aureus* (NCTC 6571) and *F. ulcerans* (NCTC 12112). ID refers to the plant collection number.
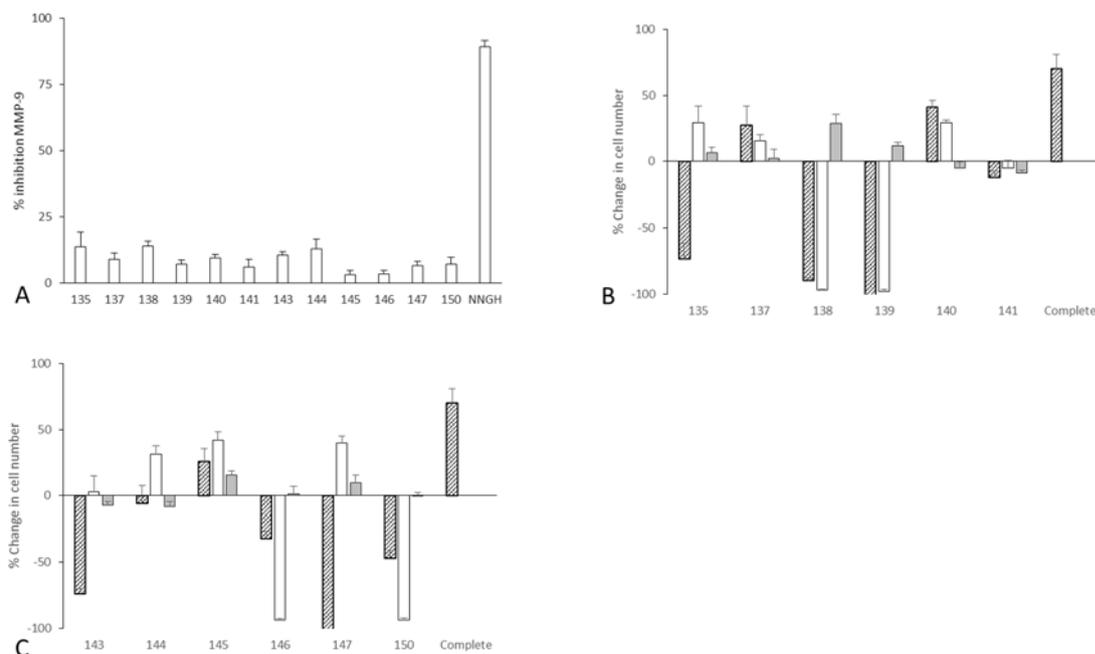

Fig. 2. Inhibition of MMP-9 by aqueous plant extracts (A). Extracts were pre-incubated at 0.01% v/v with enzyme before addition of thiopeptide substrate. Positive control is 1.3 μM NNGH. Error bars are SEM, N=6. Change in fibroblast cell number measured with CellTiter reagent after 48 hours treatment with aqueous extracts under low serum conditions (B, C). Positive control is fully supplemented complete medium. Error bars are SEM, N=4 from 4 individual experiments. Hatched bars 5% v/v, no fill 0.5% v/v, solid fill 0.05% v/v.
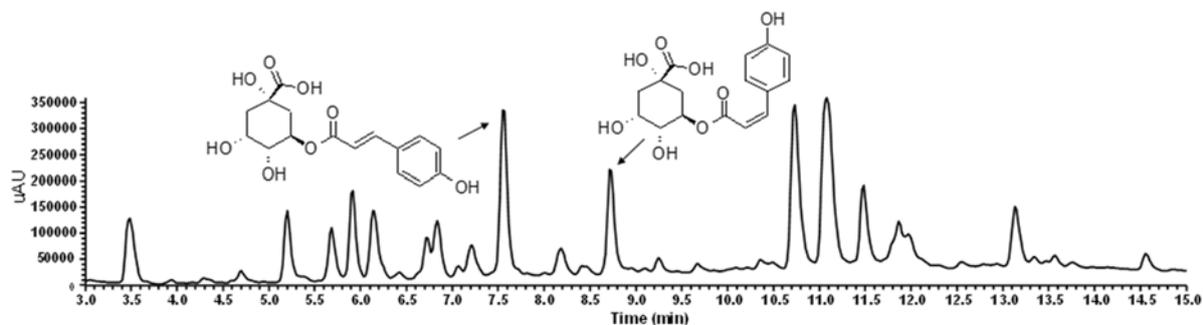
Fig. 3. HPLC-UV profile of *H. foetidum* aqueous leaf extract including two of the major components identified, trans-3-O-p-coumaroylquinic acid (RT= 7.6 min) and cis-3-O-p-coumaroylquinic acid (RT= 8.8 min).

4. Discussion and conclusion

The ethnobotanical survey identified sixteen species of plants that are used by the Apsokok community to treat tropical ulcers. Only three of these species have been recorded as tropical ulcer plant medicines in neighbouring language groups (inland Kaulong and Miu). This further reinforces the notion that medicinal plant knowledge has evolved independently in the different language groups of New Britain (Prescott et al., 2015). Nonetheless certain aspects of plant preparation are similar to those of the inland Kaulong and Miu; in particular the use of locally prepared slaked lime which three of the species are mixed with (Prescott et al., 2015; Prescott et al., 2012). Three of the plants are burnt to produce an ash which is then applied directly to tropical ulcers. This resembles the use of adsorbent activated charcoal dressings that have been used to treat chronic ulcers in developed countries (Kerihuel, 2010). The potential for carbon particles in plant ash to bind bacterial toxins or MMP enzymes should be examined further but is beyond the scope of the present study.

The most obvious way plant material could aid wound closure in a tropical ulcer is by acting as an antiseptic. Several of the plants did produce zones of inhibition with *S. aureus* but none were active against *F. ulcerans*. This is perhaps a reflection of the greater permeability of gram positive bacteria to small molecules compared with gram negative species (Lewis and Ausubel, 2006). It is worth considering that bacterial pathogens in wound exudate would experience very high concentrations of plant secondary metabolites when plant sap or moist leaf material is applied to the tropical ulcer. In such topical applications, the small zones of inhibition seen in a disc diffusion assay may translate into complete growth inhibition of bacterial pathogens in the wound environment. As the active metabolites are water soluble, this will aid their miscibility with the wound exudate and help them diffuse across the wound surface.

The wound exudate of chronic wounds is known to harbour high levels of MMP enzymes that degrade collagen and growth factors, thereby preventing normal wound progression. When assaying an enzyme in a cell free experiment it is not possible to model the very high levels of plant extract that come into direct contact with the enzyme *in vivo*. Nonetheless, using a dose of 0.01% v/v all the plants produced low levels of inhibition. In an *in vivo* context a plant sap could be present at concentrations as high as 50% v/v suggesting near complete inhibition of MMP activity could be achieved. Initially this may provide a therapeutic benefit, by for example reducing proteolytic

cleavage of collagen, but in the longer term, controlled MMP activity is necessary for wound remodelling, therefore complete inhibition may impair wound healing (Caley et al., 2015).

Another feature of the chronic wounds and infected wounds is fibroblasts that exhibit a premature senescence phenotype (Wall et al., 2008). As fibroblasts are essential for collagen deposition and growth factor secretion, plants metabolites that stimulate normal fibroblast growth in response to stress from bacterial bio-burden may help stimulate wound healing. Eight species stimulated fibroblast proliferation under low serum conditions. These effects were concentration dependent and in some cases high concentrations of plant extract induced cell death but lower doses stimulated cell proliferation. Interestingly, saps were highly represented amongst the fibroblast stimulators. The extracts of two species *Alstonia* cf. *scholaris* and *Pangium edule* induced a dose response at concentrations from 0.05 up to 5% v/v, both these species also inhibited MMP-9 raising the potential for a dual action effect.

A single species, *H. foetidum* demonstrated activity in all three of the assays. The aqueous extract of the leaf is inhibitory towards *S. aureus* in a disc diffusion assay. LC-MS revealed the presence of two coumaroylquinic acids. The related compound 3-*p-trans*-coumaroyl-2-hydroxyquinic acid displays activity towards both gram negative and positive bacteria including *S. aureus* raising the possibility that the two water soluble coumaroylquinic acids contribute towards the antibacterial activity of the extract. The *H. foetidum* extract also inhibits MMP-9 at 0.01 % v/v and stimulates the proliferation of normal adult human dermal fibroblasts at 0.05% % v/v. This suggests that when applied to a tropical ulcer, bacteria in the wound exudate will be inhibited and similarly MMP enzyme activity will be reduced; deeper in the wound where plant metabolite concertation will be lower there is also the possibility for fibroblast stimulation. For this reason *H. foetidum* merits further investigation using *in vivo* models.

Acknowledgements

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