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Evaluation of *Cypholophus macrocephalus* sap as a treatment for infected cutaneous ulcers in Papua New Guinea

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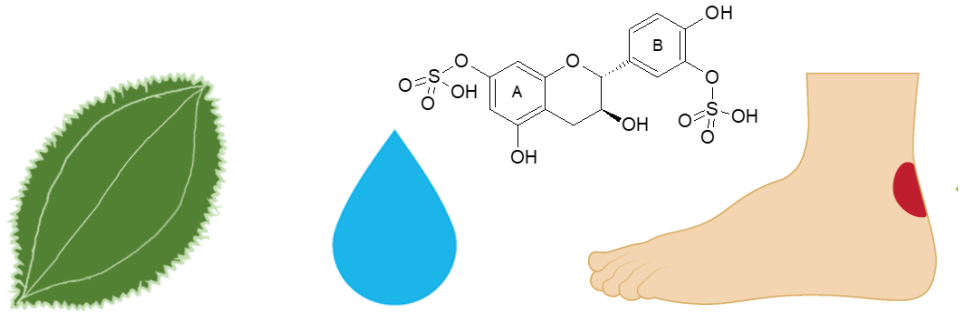
Abbreviations: ELISA (enzyme-linked immunosorbent assay); FCS (foetal calf serum); GM-CSF (granulocyte-macrophage colony-stimulating factor); IL-6 (interleukin-6), LPS (lipopolysaccharide); MMP (matrix metalloproteinase); LC-MS (liquid chromatography-mass spectrometry); PBS (phosphate buffered saline); TNF-α (tumour necrosis factor-α).

ABSTRACT

Cypholophus macrocephalus sap is used to treat bacterially infected cutaneous leg ulcers in Papua New Guinea. High resolution LC-MS analysis of the sap revealed it to be rich in sulphated flavonoids. We assessed the effects of the sap on the differentiation and pro-inflammatory anti-microbial responses of M1 macrophages using IL-6 and TNF-α ELISAs and found significant increases in M1 macrophage IL-6 expression with concentrations as low as 243 ng/ml sap. Neutrophil IL-6 and TNF-α expression was also significantly increased but to a lesser degree. Matrix metalloproteinases (MMPs) 1, 2, 8 and 9 which are known to contribute to the toxic nature of wound exudates were inhibited by the sap at 24 µg/ml. The sap was tested with several bacterial species known to colonise cutaneous ulcers in Papua New Guinea but proved not to be active. *Cypholophus* sap stimulates pro-inflammatory, anti-microbial M1 macrophage and neutrophil responses at very low concentrations, whilst also inhibiting MMPs. The combination of an enhanced innate immune response and inhibition of MMPs in ulcer exudate, may contribute to the eradication of bacteria and healing of these infected ulcers. The sap concentrations used in these assays are readily achievable in an *in vivo* context.

Graphical Abstract

Cypholophus macrocephalus sap for ulcers



M1 macrophage IL-6 simulation at 73ng/ml sap

1. Introduction

The country of Papua New Guinea, which forms the eastern half of New Guinea and its outlying islands, has been ranked second in a world ranking based on a biocultural index that takes into account a combination of biological and human cultural diversity (Loh and Harmon., 2005). Despite this, to date, of New Guinea's 1100 indigenous groups only 117 have been the subject of an ethnobotanical study (Cámara-Leret and Denneh, 2019). To the east of the island of New Guinea lies the island of New Britain, one of the longest populated tropical rainforest islands in the Pacific. Archaeological evidence from Yombon in Whitman Range that forms part of the central backbone of the island suggests human habitation there dates back at least 35,000 years (Palvides and Gosden., 1994). We have been carrying out ethnopharmacological research in the Kandrian inland area that extends into the foothills of the Whiteman Range, since 1998. Our focus has been on plants used to treat cutaneous leg ulcers, which are a highly common bacterial infection that until recently has received relatively little attention in tropical disease research (Prescott et al., 2012; Prescott et al., 2015; Prescott et al., 2017). The focus of this research is to identify promising plant saps that could be used as first-line treatments for cutaneous ulcers, in remote areas that lack access to basic health facilities.

Cutaneous skin ulcers of the lower leg are a common ailment in island Melanesia, and although relatively little studied in terms of their microbiology and pathology, they nonetheless present a significant area of unmet clinical need. Treatment options in Papua New Guinea include topical antiseptics, such as chlorhexidine; and antibiotics, such as amoxicillin; but for communities living in remote rainforest areas, access to these treatments may be limited or non-existent. For the inland Kaulong whose living conditions range from more established villages to small hamlets, and even temporary camps in rainforest clearings, access to medicine can be rather limited (Prescott et al., 2012). Even if medical help is sought in an aid post, the very process of traversing muddy forest paths to these aid posts is likely to further exacerbate the condition by submerging the infected wounds in rainforest mud. For this reason, wound healing plant saps that can be easily found in the local environment may prove to be useful first line treatments for cutaneous ulcers.

Until recently, little was known about the microbiology of cutaneous ulcers in Papua New Guinea. The classification of different types of cutaneous ulcers is still an area of ongoing research. Tropical ulcers which develop from small skin breaks, tend to be large and undermined, and have conventionally been thought of as infections involving spirochetes and fusiform bacteria, such as *Fusobacterium ulcerans* (Adriaans and Shah, 1988; Lupi et al., 2006). Early research using culture-based methods identified β-haemolytic *Streptococci*, *Staphylococcus aureus*, *Corynebacterium diphtheriae* and *Corynebacterium haemolyticum* from infected skin lesions from children in the highlands of Papua New Guinea (Montgomery, 1985). More recently, researchers applied metagenomics to cutaneous ulcers in Lihir

Island, Papua New Guinea, revealing a multitude of bacterial pathogens, such as *Haemophilus ducreyi*, *Treponema pallidum* subspecies *pertenue*, *Streptococcus dysgalactiae* and *Arcanobacterium haemolyticum* (Noguera-Julian et al., 2019).

Aside from the bacteriology of the ulcers, the host immuno-inflammatory response of the wound environment is likely to have a strong bearing on clinical outcomes with cutaneous ulcers in Papua New Guinea. In non-healing wounds prevalent in the western world, for example, diabetic ulcers and pressure sores, persistent or dysregulated inflammation is known to be key to chronicity (Sindrilaru et al., 2011; Zhao et al., 2016). Although chronic inflammation can contribute to wound persistence in the type of ulcers found in the west, an increase in pro-inflammatory responses from innate immune cells, such as macrophages and neutrophils, which are directly involved in defending against microbial pathogens could prove beneficial, if induced early on in the pathogenesis.

Here, we examine the wound healing potential of the sap of *Cypholophus macrocephalus* (Blume) Wedd., a small shrub in the Urticaceae family with a distribution ranging from Malesia to New Guinea across to the pacific islands. The sap is applied directly to cutaneous ulcers by the Kaulong speaking population of Papua New Guinea. The work presented here is to the best of our knowledge, the first phytochemical and bioactivity study of this species.

2. Materials and Methods

2.1 Ethnobotanical data collection

Approval for the ethnobotanical survey was provided by the Papua New Guinea National Research Institute, the provincial government in West New Britain and the local community itself. The ethnobotanical survey commenced in August 1998 in the village of Umbi (Kandrian Inland, West New Britain, Papua New Guinea). Initially, a putative list of cutaneous ulcer plant medicines was obtained by simply walking though rainforest with key respondents while discussing plant uses. The putative plant medicines were then discussed in greater depth in semi-structured interviews, with key respondents put forward by the community as people with a good knowledge of the local traditional medicine. During the interviews the respondents were given the opportunity to provide new plants for discussion or reject those on the list. Three different interviews were carried out with three different key respondents on three separate occasions. The interviews were carried out in Tok Pisin language by the same researcher, and were undertaken in a specific sequence that exploited the geographical separation of individual dwellings around the village of Umbi, so as to minimise cross-communication between respondents. Care was taken to frame interview questions in a way which avoided leading questions, with plant use descriptions volunteered unprompted by any previous discussion of plant use. Care was also taken to avoid over reliance on information provided by individual respondents (Etkin.,

1993) and to link local plant names back to a single field specimen (Weckerle et al., 2017). After a 19-year period, repeat ethnobotanical fieldwork was carried out in March 2017 in the same location and using the same approach, with plant use information derived from three new interviews with three new key informants.

2.2 Collection and preparation of plant material

A voucher specimen (T.A.K.P. 163) is lodged at Kew and was identified by comparison with reference material. The specimen was collected in secondary forest near the village of Umbi and temporarily preserved in 70 % methanol, before being pressed and dried. Approximately 1 ml sap from the same specimen was collected by pulverising the leaves and stem, then allowing the sap to flow into a sterile 2 ml plastic cryotube. This was carried out in the presence of key informants, according to traditional methods. This sap sample was then centrifuged at 13,400 g, filtered through a 0.02 µm PTFE syringe filter in a sterile environment and stored at -20 °C. For simplicity this processed sap sample is herein referred to as the sap. After completion of biological assays, 0.3ml of the sap were lyophilised and weighed, revealing the concentration of soluble compounds in the sap to be 24.3mg/ml.

2.3 LC-MS analysis of sap sample

The ThermoFisher Scientific liquid chromatography-mass spectrometry (LC-MS) system consisted of an 'Accela' liquid chromatograph, an Orbitrap Fusion mass spectrometer, and an electrospray source. Chromatography was achieved with a 150 mm × 3 mm × 3µm Luna C18(2) column (Phenomenex), using a 400 µl/min mobile phase gradient of 0:90:10 to 90:0:10 (water/acetonitrile/acetonitrile + 1 % formic acid) over 1 h. The mass spectrometer recorded high resolution (30 k) MS^1 spectra in positive polarity (*m*/*z* 125-1800), low resolution MS^1 spectra in negative polarity (*m*/*z* 125-1800); and data directed serial mass spectra (MS^2 and MS^3, 4 *m*/*z* isolation width, 35 % energy) in both polarities.

2.4 Antibacterial assays

MIC broth dilution and disc diffusion antibacterial assays were carried out as described previously (Moses et al., 2020). The organisms used were *Staphylococcus aureus* (NCTC 6571), *Fusobacterium ulcerans* (NCTC 12112) and *Streptococcus pyogenes* (NCTC 8198) (Prescott et al. 2017; Moses et al 2020). Positive control discs contained 15 µl 10 % w/v povidone iodine (Vetasept) or 0.2 % chlorhexidine. Zones of inhibition were obtained by averaging four different measurements per disc, with individual experiments carried out in duplicate.

2.5 Matrix metalloproteinase inhibition assay

Sap effects on MMP-1, -2, -8 and -9 activities were carried out as described (Moses et al., 2020), using the MMP Inhibitor Profiling Kit, Fluorometric Assay (Enzo Life Sciences, Exeter, UK), scaled down to a final reaction volume of 25 µl for a 384-well plate format. Preliminary experiments were undertaken

to determine the highest sap concentration that could be used without quenching fluorescence from the MMP reaction product. The positive control N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH) was used at 1.3µM.

2.6 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*. (Moseley et al., 2003), as described recently (Moses et al., 2020). Whole blood (in 20 ml aliquots) was collected from healthy, human volunteers (age range 20-40 years).

2.7 Pro-inflammatory cytokine release by human neutrophils and M1 macrophages

Pro-inflammatory cytokine quantification was carried out, as described previously (Moses et al., 2020). Briefly, isolated neutrophil or macrophage cultures ($5x10^5$ cells/ml) were established in the absence and presence of sap (at sub-lethal concentrations of 0.001 %, 0.01 % and 0.1 %). 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.

2.8. Dermal fibroblast and keratinocyte proliferation

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

2.9. Statistical analysis

Results are expressed 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. Significance were considered at $p<0.05$.

3. Results

3.1 Cypholophus macrocephalus is applied topically to treat cutaneous ulcers by the inland Kaulong population of Papua New Guinea

Ethnobotanical research was initiated with the inland Kaulong community in 1998, with follow up work completed in 2017. Careful questioning of key ethnobotanical respondents over this large time period provided data from six respondents consistently supporting the use of the sap of this species as a topical treatment for cutaneous ulcers. The plant use is further supported by a previous report of *Cypholophus*

stem being used to treat ulcers in Waigeo Island in Papua Province Indonesia (Susiarti et al., 2018). In our study, first-hand observations of sap preparation revealed that the soft leaves and stems of the plant are pulverised in the palm of the hand by rubbing both hands together. The resulting ball of pulverised plant material is then squeezed to provide sap that is then allowed to drip directly onto the ulcer surface.

3.2 The sap sample contains epicatechin-O-disulphate and two isomers of quercetin-O-sulphate

High resolution LC-MS analysis of the sap (Fig. 1), led to a tentative identification of three major compounds based on their negative ESI-MS/MS fragmentation patterns, revealing the sap to be rich in sulphated flavonoids.

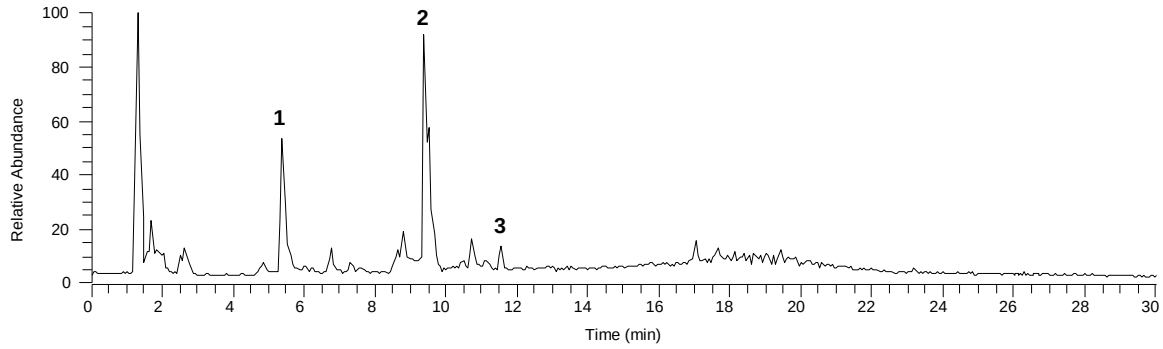
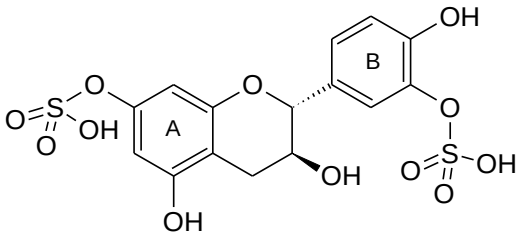
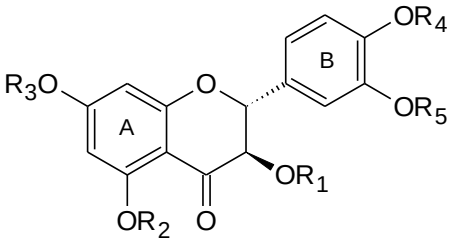


Fig. 1. Sulphated flavonoids (**1**~**3**) detected from the sap sample by high resolution LC-MS in negative mode.



1. Catechin-disulphate (tentative identification).



2 and **3** are two of five potential isomers of quercetin-*O*-sulphate where one R= sulphate and the remaining R = H.

Epi/catechin-*O*-disulphate (**1**) and two quercetin-*O*-sulphates (**2** and **3**) were detected as major components by mass spectrometry in negative mode, from the neutral loss after collision induced dissociation (Kleinenkuhnen et al., 2019). The neutral loss of 80 Da corresponding to the loss of a SO_3 group was observed for each of the compounds. The identity of the aglycone in each compound was confirmed by comparison of their MS3 spectra with the characteristic mass spectra obtained from standard compounds. The aglycone of (**1)** was determined to be catechin or epicatechin by comparison of its MS3 fragment *m/z* 289 with MS2 of a standard catechin. The two sulphate groups were determined to be attached to the A-ring and B-ring of the flavanol separately. Key fragments (*m/z*: 231 and 217) for such an arrangement were observed for **1**, as previous work has demonstrated that MS2 fragmentation patterns with an ion at *m/z* 217 (137 + 80) indicates a sulphate residue linked to the epi/catechin A-ring whereas the ion at *m/z* 231 (151 + 80) indicate it is linked to B-ring (Gonzalez-Manzano., 2009). The MS3 spectra of *m/z* 301 in (**2)** and (**3)** match the spectrum of a quercetin standard, indicating they are monosulphate isomers with the sulphate group attached to two different hydroxyl positions on the quercetin moiety.

Epi/catechin-*O*-sulphate (**1**). Rt 5.39 min, LC-ESI-MS (negative mode) *m/z*: 449 $[M-H]^-$; ion trap MS/MS of m/z 369 $[M-SO3H]^-$, *m/z* (rel. int.): 289(100), 245(12), 231(36), 217(62), 137(15). HR-ESI-MS m/z: 448.9849 $[M-H]^-$, calculated for $C_{15}H_{13}O_{12}S_2$: 448.9848.

Quercetin-*O*-sulphate A (**2**) Rt 9.41 min, UV (LC PDA) λmax (nm): 254, 353; LC-ESI-MS (negative mode) m/z: 381 $[M-H]^-$, HR-ESI-MS *m/z*: 380.9916 $[M-H]^-$, calculated for C15H9O10S: 380.9916.

Quercetin-*O*-sulphate B (**3**) Rt 11.54 min, UV (LC PDA) λmax (nm): 266, 352; LC-ESI-MS (negative mode) *m/z*: 381 $[M-H]^-$, HR-ESI-MS *m/z*: 380.9917 $[M-H]^-$, calculated for $C_{15}H_9O_{10}S$: 380.9916. Both quercetin-*O*-sulphate isomers produced ion trap MS/MS of the aglycone *m/z* 301 $[M-SO3H]^-$: 273(24), 257(20), 229(6), 193(5), 179(100), 151(75), 107(3) that match the fragmentation pattern of a quercetin standard.

3.3 The sap does not inhibit a panel of bacterial species known to infect skin ulcers in New Guinea

S. aureus is known to colonize cutaneous ulcers in Papua New Guinea (Montgomery, 1985). A drug-sensitive strain *S. aureus* 6571 was used with high concentrations of the sap 6 % v/v. As a gram-positive species this strain is sensitive to a wide variety of antibacterial compounds. Despite the high sap concentration used, no significant reductions in bacterial growth were observed, compared to untreated controls, suggesting the sap lacks significant antibacterial activity. Next, the sap was tested using disc diffusion assays against the anaerobic gram-negative wound pathogens, *F. ulcerans*; and the aerobic gram-positive species, *S. aureus* and *S. pyogenes*. These pathogens were selected as non-fastidious organisms that are known to infect cutaneous ulcers in Papua New Guinea (Montgomery, 1985;

Adriaans and Shah, 1988; Noguera-Julian et al., 2019). The topical antiseptics chlorhexidine and povidone iodine were also included as positive controls. A high dose of test sample per disc (15µl) was used to help reveal zones of inhibition. A disc diffusion assay was used as this most closely mimics the application of the sap to the surface of the wound, where antibacterial activity is dependent on the diffusion of active compounds across the wound surface. The results (see Table 1.) demonstrate that the sap shows no significant inhibitory activity towards these three pathogens. Positive controls displayed activity as expected.

	S. aureus	*S. pyogenes*	*F. ulcerans*
Cypholophus sap	0	0	0
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 disc diffusion assays. Each 6 mm paper disc was treated with 15 µl plant sap, 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). Additionally, no inhibition was observed in a broth microdilution assay with *S. aureus* 6571 at 6 % v/v. Povidone and chlorhexidine results were obtained as part of a multiple sample experiment carried out in parallel and reported previously (Moses et al., 2020).

3.4 The sap inhibits matrix metalloproteinase enzyme activity

Wound exudates containing excessive amounts of matrix metalloproteinase (MMP) enzymes has been linked to the non-healing status of chronic wounds, due to the ability of MMPs to degrade various extracellular matrix components and growth factors. In particular, MMP-1, MMP-2, MMP-8 and MMP-9, are known to be elevated in chronic wound environments, with elevated MMP activities correlating with delayed healing (Lazaro et al., 2016). Therefore, polar compounds from the plant sap that are readily soluble in the wound exudate could prove beneficial in an *in vivo* context, if they inhibit MMP enzyme activity. The sap was tested with four different MMP enzymes using a quenched fluorogenic peptide substrate in a cell-free assay (Fig. 2.). Figure 2. shows that 0.1 % v/v sap (the highest concentration permissible with the assay), acts to inhibit all four MMP isoforms assessed. This corresponds to a concentration of 24 µg/ml sap metabolites. In an *in vivo* context, the sap could be present in the wound exudate at much high concentrations and therefore, potentially provide higher levels of inhibition.

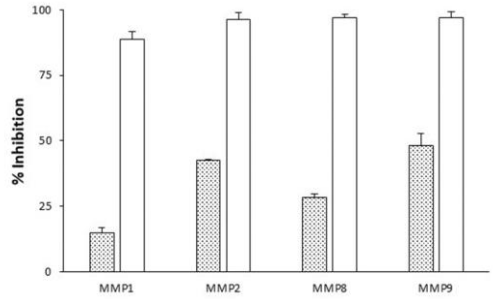


Fig 2. Effect of 0.1% v/v/ sap on MMP-1, -2, -8 and -9 activities in a cell-free assay. Shaded bars indicate sap sample, un-shaded bars are control inhibitor 1.3µM NNG. Error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments.

3.5 The sap increases TNF-α and IL-6 secretion from isolated human neutrophils and macrophages in a dose-dependent manner

The effects of the sap on the activation and pro-inflammatory responses of isolated human neutrophils and M1 macrophages were subsequently assessed, by quantifying TNF-α and IL-6 secretion into culture medium by ELISA. Patient-derived primary cells were used as they closely resemble the status of these cell types *in vivo*. For neutrophils, (Fig. 3.), negative and positive controls with and without LPS were included for comparison, but the response to the sap was determined in the absence of LPS. Macrophage response to the sap was determined in the presence of GM-CSF, to stimulate the polarisation and activation of the pro-inflammatory M1 macrophage sub-type responsible for counteracting infection (Fig. 4.). Controls containing no GM-CSF and the highest sap concentration (0.1 %) were also included for comparison, as were controls with and without GM-CSF in the absence of sap.

With the neutrophils, TNF-α and IL-6 secretion was significantly stimulated in a dose-dependent manner. For IL-6, maximal detection equivalent to LPS controls was achieved at 0.01 % sap concentrations ($p<0.001$ versus untreated controls). This corresponds to a concentration of sap metabolites of 2.4 µg/ml, and was further evident at 0.1 % v/v. TNF-α responses were less induced, but still followed a clear dose-response relationship, with significant increases in TNF-α secretion equivalent to LPS controls determined at 0.1 % sap concentrations ($p<0.001$).

For the macrophages, IL-6 levels were already maximal at 0.001 % v/v sap ($p<0.001$), which corresponds to a concentration of sap metabolites of just 243 ng/ml. TNF-α secretion was again less

sensitive, but also followed a clear dose-response relationship with significant increases detected at 0.01 % and 0.1 % v/v sap (both $p<0.001$). The 0.01 % v/v sap concentration corresponds to 2.4 µg/ml sap metabolites. Additionally, for both IL-6 and TNF-α, significant increases in release are achieved at 0.1 % sap concentrations (24 µg/ml), in the absence of GM-CSF. Thus, such stimulatory effects on cytokine secretion is GM-CSF independent, indicative of its potential abilities in promoting the polarisation and activation of the pro-inflammatory M1 macrophage sub-type responsible for counteracting infection.

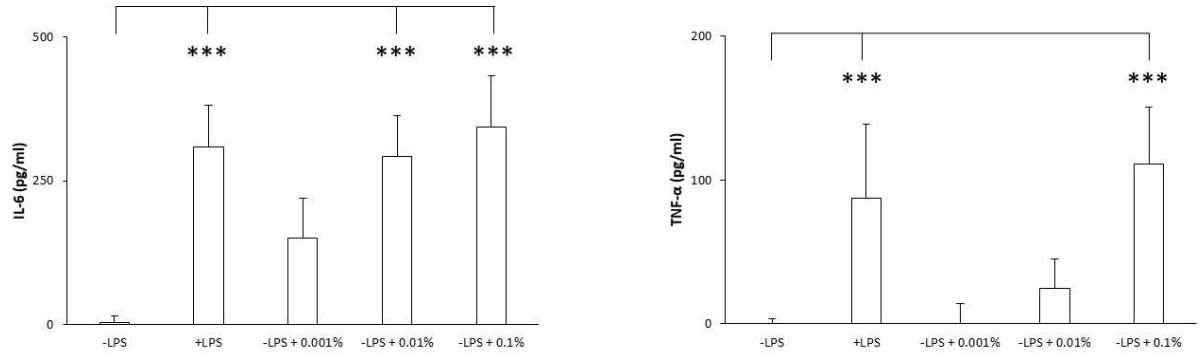


Fig 3. Sap effects on the stimulation of IL-6 and TNF-α secretion by isolated human neutrophils. % indicates % v/v of sap. Controls -LPS and +LPS indicate cell responses with and without 10 µg/ml LPS, in the absence of sap. Bars marked 0.001 %, 0.01 %, 0.1 % are sap concentrations in % v/v as indicated, in absence of LPS. For both graphs, error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments.

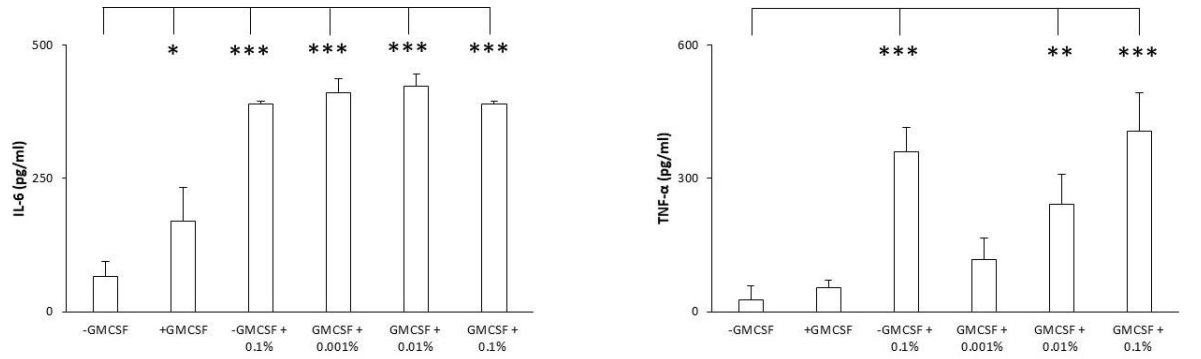


Fig 4. Sap effects on the stimulation of IL-6 and TNF-α secretion by M1 macrophages. % indicates % v/v of sap. Controls –GM-CSF and +GM-CSF indicate cell responses with and without GM-CSF 10 ng/ml, in the absence of sap. –GM-CSF + 0.1 % indicates response to 0.1 % sap in absence of GM-CSF. Bars marked 0.001 %, 0.01 % and 0.1 % are GM-CSF-stimulated cells with sap concentrations in

% v/v as indicated. For both graphs, error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments.

3.6 The sap does not stimulate fibroblast or keratinocyte proliferation

In normal wound healing, fibroblasts mediate wound closure, by secreting growth factors and depositing extracellular matrix components, enabling wound re-epithelialisation by keratinocytes (Werner et al., 2007; Barrientos et al., 2008). However, fibroblasts isolated from non-healing chronic wounds possess significantly impaired proliferative, migratory, matrix turnover and growth factor responsiveness capabilities, due to the increased onset of cellular senescence within chronic wound environments (Wall et al., 2008). We were interested in determining the effects of the sap on fibroblast and keratinocyte proliferation. Fig. 5. shows that both fibroblasts and keratinocytes show no significant increases in proliferation in response to the sap (all $p>0.05$).

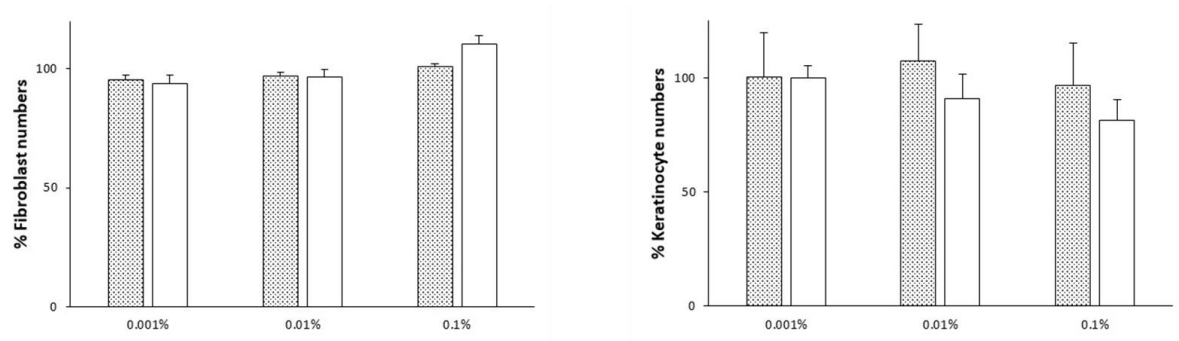


Fig 5. Effect of the sap on dermal fibroblast and keratinocyte proliferation at 24 h (shaded) and 72 h (unshaded), relative to untreated controls. Error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments.

4. Discussion

Sulphated flavonoids have been previously identified from certain plant families, such as Arecaceae, Asteraceae, Bixaceae, Dilleniaceae, Frankeniaceae, Gramineae, Guttiferae, Juncaceae, Malvaceae, Tamaricaceae, Umbelliferae and Verbenaceae (Teles et al., 2018). Quercetin-*O*-sulphates (including single and multi-sulphate esters) have been frequently observed in the Asteraceae (Harborne and Baxter, 1999). Although epi/catechin-*O*-sulphate has been detected as a metabolite in the human body after

consumption of dietary flavonoids, epi/catechin-*O*-sulphate has not to the best of our knowledge been previously found directly in a plant. Additionally, as far as we are aware, this is the first time that sulphated flavonoids have been reported in the family Urticaceae. Further work should be carried out with plant material from different sources to isolate these compounds and confirm their identity.

Our previous work on Papua New Guinea plant medicines used for cutaneous leg ulcers, including tropical ulcers, suggests that not all plants used for this therapeutic indication possess antibacterial activity (Prescott et al., 2012; Prescott et al., 2017). However, the complexity of wound healing biology is such that a wide range of modes of action, unrelated to antibacterial activity, could potentially contribute to *in vivo* efficacy.

One possible mechanism through which bacterial infection could be reduced is by stimulation of the innate immune response. There is some precedent this; the macrophage stimulating agent, recombinant GM-CSF has been trialled as a topical therapy for non-healing ulcers with some limited indication of success. (Huang et al., 2014). However, it is not clear to what extent enhanced clearance of bacterial bioburden is involved in the improved healing rates observed (Khan and Davies, 2006). Nonetheless, the highly potent activity of the sap, which significantly stimulated both macrophage and neutrophil activation at just 2.4 µg/ml, makes the use of this agent on open wounds intriguing. As the sap is applied directly to the wound surface, sap concentrations used in this study would be readily achieved *in vivo*.

However, any potential benefits derived from enhanced pro-inflammatory responses must be balanced against the downstream effects of elevated IL-6 and TNF-α, which also act to induce increased expression of MMPs. Elevated MMP levels result in increased degradation of wound collagen and extracellular matrix components, which in turn results in stalled wound healing (Barrientos et al., 2008). Nonetheless, agents such as *Cypholophus* sap that stimulate the innate immune response while simultaneously inhibiting MMP enzymes in the wound exudate deserve further consideration. This is especially the case for polar/water soluble compounds such as those in the sap that can easily diffuse in the aqueous wound exudate without being absorbed deeper into the wound, where inhibition of normal MMP function might impair wound remodelling. Thus, by inhibiting the excessive proteolytic activity within these wounds, degradation of growth factors and extracellular matrix components could be reduced, resulting in improved healing rates. Further work should be carried out with isolated *Cypholophus* sap metabolites using *in vivo* models of infected wounds, in order to further asses their therapeutic potential.

Declaration of competing interests

The authors declare no competing interests.

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References

Adriaans, B., Shah, H., 1988. Fusobacterium ulcerans Sp. nov. from tropical ulcers. Int. J. Syst. Bacteriol. 38, 447-448.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., Tomic-Canic, M., 2008. Growth factors and cytokines in wound healing. Wound Rep. Regen. 16(5), 585-601.

Cámara-Leret, R., Dennehy, Z., 2019. Indigenous knowledge of New Guinea's useful plants: A review. Econ. Bot.., 73(3), 405-415.

Etkin, N.L., 1993. Anthropological methods in ethnopharmacology. J. Ethnopharmacol. 38, 93–104.

Gonzalez-Manzano, S., Gonzalez-Paramas, A., Santos-Buelga, C., & Duenas, M. 2009. Preparation and characterization of catechin sulfates, glucuronides, and methylethers with metabolic interest. J Agricultural Food Chem, 57(4), 1231-1238.

Harborne J. B., Baxter H., 1999. The Handbook of Natural Flavanoids. Wiley: Chichester, UK. 1999; (1) 264-271.

Huang, G., Sun, T., Zhang, L., Wu, Q., Zhang, K., Tian, Q., Huo, R., 2014. Combined application of alginate dressing and human granulocyte-macrophage colony stimulating factor promotes healing in refractory chronic skin ulcers. Exp. Ther. Med. 7(6), 1772-1776.

Khan, M., Davies, C., 2006. Advances in the management of leg ulcers-the potential role of growth factors. Int. Wound J. 3:113-120.

Kleinenkuhnen, N., Buchel, F., Gerlich, S. C., Kopriva, S., & Metzger, S., 2019. A novel method for identification and quantification of sulfated flavonoids in plants by neutral loss scan mass spectrometry. Front Plant Sci., 10, 885.

Lazaro, J.L., Izzo, V., Meaume, S., Davies, A.H., Lobmann, R., Uccioli, L., 2016. Elevated levels of matrix metalloproteinases and chronic wound healing: an updated review of clinical evidence. J. Wound Care 25(5), 277-287.

Loh, J., Harmon, D., 2005. A global index of biocultural diversity. Ecol. Indic. 5, 231–241.

Lupi, O., Madkan, V., Tyring, S.K., 2006. Tropical dermatology: Bacterial tropical diseases. J. Am. Acad. Dermatol. 54, 559–578.

Montgomery, J., 1985. The aerobic bacteriology of infected skin lesions in children of the Eastern Highlands Province. PNG Med. J. 28(2), 93-103.

Moseley, R., Walker, M., Waddington, R.J., Chen, W.Y., 2003. Comparison of the antioxidant properties of wound dressing materials - carboxymethylcellulose, hyaluronan benzyl ester and hyaluronan, towards polymorphonuclear leukocyte-derived reactive oxygen species. Biomaterials 24(9), 1549-1557.

Moses, R.L., Dally, J., Lundy, F,T., Langat, M., Kiapranis, R., Tsolaki, A.G., Moseley, R., Prescott, T.A.K., 2020. Lepiniopsis ternatensis sap stimulates fibroblast proliferation and down regulates macrophage TNF-α secretion. Fitoterapia 141, 104478. Advance online publication. doi.org/10.1016/j.fitote.2020.104478

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65(1-2), 55-63.

Noguera-Julian, M., Gonzalez-Beiras, C., Parera, M., Ubals, M., Kapa, A., Paredes, R., Mitja, O., 2019. Etiological characterization of the cutaneous ulcer syndrome in Papua New Guinea using shotgun metagenomics. Clin. Infect. Dis. 68(3), 482-489.

Palvides, C., Gosden, C., 1994. 35,000-Year-old sites in the rainforests of West New Britain, Papua New Guinea. Antiquity 68, 604–610.

Prescott, T.A., Kiapranis, R., Maciver, S.K., 2012. Comparative ethnobotany and in-the-field antibacterial testing of medicinal plants used by the Bulu and inland Kaulong of Papua New Guinea. J. Ethnopharmacol. 139, 497–503.

Prescott, T.A., Briggs, M., Kiapranis, R., Simmonds, M.S., 2015. Medicinal plants of Papua New Guinea's Miu speaking population and a focus on their use of plant-slaked lime mixtures. J. Ethnopharmacol. 174, 217-223.

Prescott, T.A.K., Homot, P., Lundy, F.T., Fang, R., Patrick, S., Camara-Leret, R., Kiapranis, R., 2017. Tropical ulcer plant treatments used by Papua New Guinea's Apsokok nomads. J. Ethnopharmacol. 205, 240-245.

Sindrilaru, A., Peters, T., Wieschalka, S., Baican, C., Baican, A., Peter, H., Hainzl, A., Schatz, S., Qi, Y., Schlecht, A., Weiss, J.M., Wlaschek, M., Sunderkotter, C., Scharffetter-Kochanek, K., 2011. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. J Clin Invest 121(3), 985-997.

Susiarti, S., Arifiani, D., Ibo, L., 2018. Knowledge and usage of medicinal plants by local people in Waigeo Island, Raja Ampat, West Papua, Indonesia. Drug Invention Today 10 (5), 3785-3790.

Teles Y. C. F., Souza, M. S. R., Souza M. F. V., 2018. Sulphated flavonoids: Biosynthesis, structures, and biological activities. Molecules 23, 480.

Wall, I.B., Moseley, R., Baird, D.M., Kipling, D., Giles, P., Laffafian, I., Price, P.E., Thomas, D.W., Stephens, P., 2008. Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. J. Invest. Dermatol. 128(10), 2526-2540.

Weckerle, C. S., de Boer, H. J., Puri, R. K., van Andel, T., Bussmann, R. W., & Leonti, M., 2018. Recommended standards for conducting and reporting ethnopharmacological field studies. J Ethnopharmacol, 210, 125-132.

Werner, S., Krieg, T., Smola. H., 2007. Keratinocyte-fibroblast interactions in wound healing. J. Invest. Dermatol. 127(5), 998-1008.

Xu, R., Sun, H.F., Williams, D.W., Jones, A.V., Al-Hussaini, A., Song, B., Wei, X.Q., 2015. IL-34 Suppresses Candida albicans induced TNF-α production in M1 macrophages by downregulating expression of dectin-1 and TLR2. J. Immunol. Res., 328146.

Zhao, R., Liang, H., Clarke, E., Jackson, C., Xue, M., 2016. Inflammation in chronic wounds. Int. J. Mol. Sci. 17(12), pii: E2085.

Conflict of interest statement

Declarations of interest: none.