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Lepiniopsis ternatensis sap stimulates fibroblast proliferation and down regulates macrophage TNF-α secretion

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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); NMR (nuclear magnetic resonance); PBS (phosphate buffered saline); TNF-α (tumour necrosis factor-α).

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ABSTRACT

The sap of the tree *Lepiniopsis ternatensis* is used as a topical treatment for cutaneous leg ulcers in Papua New Guinea. This study, which is the first investigation of this medicinal plant, examines the effect of the sap on wound healing biology using human-derived primary cell lines. NMR spectra from 1D and 2D experiments revealed the sap to contain a single major component, identified as the polyphenol, trifucol. The sap significantly increased the proliferation of dermal fibroblasts at just 1.3 µg/ml, without influencing keratinocytes, suggesting a fibroblast-specific mechanism of stimulation. It also significantly inhibited TNF-α secretion by pro-inflammatory M1 macrophages, but not from neutrophils, at 130 µg/ml. The low toxicity of the sap towards dermal cells along with its fibroblast stimulation activity and downregulation of macrophage TNF-α makes it a potentially attractive agent to promote dermal wound healing in chronic non-healing ulcers.

1. Introduction

In the present study, we examine the sap from *Lepiniopsis ternatensis* Valeton (Apocynaceae), which is used by the Kaulong speaking population of Papua New Guinea as a treatment for infected cutaneous ulcers. The sap is obtained by cutting the tree and is applied directly to leg ulcers or tropical ulcers in the same manner as a topical antiseptic. *Lepiniopsis ternatensis* is a medium-sized tree growing up to 36 meters tall and contains copious amounts of white sap (Middleton, 2007). Its distribution ranges from the Philippines, Sulawesi, Moluccas, New Guinea through to the Bismarck Archipelago, where it grows in a variety of forest types up to 300m above sea level. The medicinal use of the tree has not been reported in our previous ethnobotanical surveys of other language groups in West New Britain (Prescott et al., 2012; Prescott et al., 2015; Prescott et al., 2017). Furthermore, very little has been reported on the phytochemistry of *Lepiniopsis ternatensis*; there is just a single report of unsubstituted coumarin being present in the bark and leaves (Johns et al., 1968).

Cutaneous skin ulcers of the lower leg are common in children living in rural Papua New Guinea and present a significant area of unmet clinical need. Treatment options include antiseptics and antibiotics, but a lack of access to basic healthcare often precludes these options. For the inland Kaulong, who live in small scattered hamlets in clearings in the rainforest, access to medicine is limited (Prescott et al., 2012). The area is mountainous and making a journey to an aid post involves arduous trekking through rugged terrain. Thus, there is a compelling rationale for early treatment of small cutaneous ulcers with a locally available plant, rather than late treatment of chronic ulcers with an antibiotic in a clinic. For this reason, we are carrying out research to identify wound healing plant saps in Papua New Guinea. The most promising plant saps will be evaluated in clinical trials in Papua New Guinea, the eventual aim being to identify a clinically effective phytotherapy agent for use as a first line treatment in remote areas that lack basic healthcare.

The bacterial flora of cutaneous ulcers in the South Pacific is a continuing area of research and it is still not fully understood which organisms are essential for the lesions to develop; and how the ulcers may be classified into different groups. Tropical ulcers which develop from small scratches into undermining ulcers have traditionally been viewed as polymicrobial infections involving spirochetes and fusiform bacteria, such as *Fusobacterium ulcerans* (Aadrians and Shah, 1988; Lupi et al., 2006). A 1980s study of infected skin lesions from children living in the highlands of Papua New Guinea identified β-haemolytic *Streptococci*, *Staphylococcus aureus*, *Corynebacterium diphtheriae* and *Corynebacterium haemolyticum* as the major wound pathogens (Montgomery, 1985). More recently, *Haemophilus ducreyi* has been identified from cutaneous ulcers in the Solomon Islands (Marks et al., 2014). A 2018 study that applied metagenomics to cutaneous ulcers in Lihir Island, Papua New Guinea, revealed the presence of a wide range of bacterial species such as *Haemophilus ducreyi*, *Treponema pallidum* subspecies *pertenue*, *Streptococcus dysgalactiae* and *Arcanobacterium haemolyticum* (Noguera-Julian et al., 2019).

Research on non-healing chronic wounds prevalent in westernised societies, such as venous ulcers, diabetic ulcers and pressure sores, has demonstrated the connection between persistent or dysregulated inflammation and their non-healing state (Zhao et al., 2016). Although these ulcers are distinct from the bacterial skin ulcers found in Papua New Guinea, they are likely to have certain fundamental aspects of wound biology in common. In these types of ulcers, chronic inflammation is known to give rise to a non-healing condition in which wound progression is stalled at the early inflammatory phase. Pro-inflammatory neutrophils and M1 macrophages which predominate during early dermal healing continue to persist, resulting in non-healing, chronic ulcers (Eming et al., 2007). Furthermore, elevated levels of TNF-α derived from neutrophils and macrophages, acts to induce increased expression of matrix metalloproteinases (MMPs) which degrade wound collagen and extracellular matrix components (Moseley et al., 2004; Barrientos et al., 2008). Additionally, fibroblasts from chronic wounds are well-established to exhibit significantly impaired proliferative, migratory and other responses, compared to those derived from healthy skin, as a consequence of enhanced cellular senescence within chronic wound environments (Wall et al., 2008). Thus, despite the lack of research into the pathogenesis of infected cutaneous ulcers in Papua New Guinea, studies of medicinal plants for these ulcers can make use of assays determined to be relevant to other types of skin ulcers.

2. Materials and methods

2.1. Ethnobotanical data collection

Permission to carry out an ethnobotanical survey was granted by the government of Papua New Guinea with secondary approval from local level government in West New Britain, along with permission from the local community itself. Ethnobotanical survey work was initially carried out in August 1998 in the village of Umbi in the Kandrian Inland region of West New Britain, Papua New Guinea. Informal

rainforest walks with key respondents were used to establish a range of plant medicines and uses. Plant use data were then discussed in greater depth in semi-structured interviews. The interviews were carried out three times taking care to ensure different individuals were present during each interview. Timing and sequencing of interviews combined with the geographical separation of micro-communities around the village of Umbi was used to minimise cross-communication between interview groups. In all cases, interviews were carried out in Tok Pisin language (Neo-Melanesian). Interview questions were framed so as to avoid leading questions, with key ethnobotanical data volunteered by respondents themselves, unprompted by any previous discussion of plant use. Repeat ethnobotanical fieldwork was carried out in March 2017 in the same location, with two additional respondents, using the same interview approach.

2.2. Collection and preparation of plant material

Voucher specimens were collected from forest surrounding the village of Umbi in the presence of key informants. Specimens were preserved in 70 % methanol before pressing and drying. A botanical voucher (T.A.K.P. 155) is lodged at Kew, and was identified as *Lepiniopsis ternatensis* Valeton by Apocynaceae specialist David Middleton. A small sample of the sap (approx. 1 ml) was collected by cutting the stems of the tree and letting the sap run into a sterile 2 ml plastic cryotube. The sap sample which contained solidified material was later re-constituted into sterile distilled maintaining the original sample volume. This sample was then centrifuged at 13,400 g, filtered through a 0.02 µm PTFE syringe filter under sterile conditions and stored at -20 °C. Lyophilisation of 330µl of the sample revealed a concentration of soluble metabolites of 12.97mg/ml. This re-constituted aqueous sap sample is herein referred to as the sap for simplicity.

2.3. NMR analysis of sap sample

The sap was lyophilised and re-suspended in distilled water. NMR spectra from 1D and 2D experiments were obtained using a Brucker Advance 400 Mhz instrument (Bruker UK, Coventry, UK).

2.4. Antibacterial assays

Antibacterial disc diffusion assays were carried out as described previously (Prescott et al. 2017). Briefly, *Fusobacterium ulcerans* (NCTC 12112) was cultured in an anaerobic cabinet at 37 °C, in anaerobic broth (Lab M, Bury, UK) and adjusted to OD_{600} 0.08-0.13 (0.5 McFarland score). Nutrient agar plates (Lab M) were inoculated with 200 µl culture and fully dried before adding four sterile discs (Whatman Grade AA, 6mm diameter, Sigma, Poole, UK), containing 15 µl of sterile filtered plant extract or positive control. Positive control discs contained 15 µl 10 % w/v povidone iodine (Vetasept) or 0.2 % chlorhexidine. Plates were incubated for three days in an anaerobic cabinet, before zones of inhibition were determined by taking an average of four different measurements of zone diameters, subtracting the disc diameter and then dividing by two. *Staphylococcus aureus* (NCTC 6571) was

grown and treated as outlined above, but using aerobic conditions and Mueller Hinton broth and solid media (Oxoid, Basingstoke, UK). *Streptococcus pyogenes* (NCTC 8198) was cultured aerobically at 37 °C in Mueller Hinton broth and on Columbia blood agar plates (Oxoid). The broth microdilution assay was carried out in 96-well plates with serial dilutions of the sap starting at 6% v/v in a final volume of 150 µl. *Staphylococcus aureus* (NCTC 6571) was diluted to be equivalent to a 0.5 McFarland standard and the plate was examined for growth inhibition after 24 h incubation at 37 °C.

2.5. Matrix metalloproteinase inhibition assay

The MMP-1, -2, -8 and -9 assays were carried out 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. The assay was performed as per Kit instructions with fluorescence read at 545 nm/576 nm in a Tecan M200 plate reader (Tecan Life Sciences, Reading, UK). Experiments were performed on n=3 independent occasions, with data expressed as % inhibition relative to untreated controls.

2.6. Isolation of neutrophils and macrophages from whole human blood

Neutrophils and non-polarised macrophages were isolated from whole blood, based on the method of Moseley *et al*. (Moseley et al., 2003). Briefly, whole blood was collected from healthy, human volunteers (age range 20-40 years). Blood aliquots (20 ml) were layered into 50 ml conical tubes, containing lower layers of 10 ml Histopaque®-1119 and upper layers of 10ml Histopaque®-1077 (both Sigma, Poole, UK). Tubes were centrifuged (700 g, 30 min) at room temperature, with neutrophil- and macrophage-rich layers collected and pooled separately in fresh conical tubes.

For neutrophil isolation, equal volumes of sterile phosphate buffered saline (PBS) were added to each tube and centrifuged (700 g, 5 min) at room temperature. Supernatants were discarded and erythrocyte contamination removed by the repeated addition of cold 0.2 % sodium chloride (2.5 ml, Fisher Chemicals, Loughborough, UK) to each cell pellet for 1 min. The osmolarity within each tube was subsequently corrected by the addition of 1.6 % sodium chloride (2.5 ml) to each tube, with gentle agitation. Cell pellets were washed once in PBS (5 ml), centrifuged (700 g, 5 min) and re-suspended in RPMI-1640 medium, supplemented with L-glutamine (2 mM) and 10 % heat-inactivated, foetal calf serum (FCS) (all ThermoFisher Scientific, Paisley, UK), prior to assessment of cell viability using Trypan Blue (Sigma) and cell counting.

For macrophage isolation, equal volumes of sterile PBS were added to each tube and centrifuged (380 g, 10 min) at room temperature. Supernatants were discarded and cell pellets washed once in PBS (50 ml), re-centrifuged (200 g, 10 min); and re-suspended in RPMI-1640 medium, supplemented with L-glutamine (2 mM) and 10 % heat-inactivated FCS. Cell suspensions (50 µl) were mixed with 2 % acetic acid (450 µl, Fisher Chemicals) to lyse remaining erythrocytes, prior to cell viability assessment using Trypan Blue and cell counting.

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

Isolated neutrophil cultures ($5x10^5$ cells/ml) were established in RPMI-1640 medium, with L-glutamine (2mM) and 10% heat-inactivated FCS, in the absence and presence of sap (at sub-lethal concentrations of 0.01 %, 0.1 % and 1 %). Cultures were subsequently stimulated with lipopolysaccharide (LPS, 10 µg/ml, from *E. coli*, 055:B5, Sigma). Control cultures included unstimulated (LPS-free) neutrophils and neutrophils with the highest concentration of sap (1 %), but without LPS stimulation (to confirm the absence of sap pro-inflammatory effects). Cultures were maintained at 37 °C/5 % CO_2 for 24 h. Neutrophil conditioned media was subsequently removed, centrifuged (8,000 g, 5 min); and IL-6 and TNF-α levels quantified using commercial Quantikine ELISAs (R&D Systems, Abingdon, UK) and a Bio-Tek Instruments Microplate Autoreader EL311 (ThermoFisher Scientific), at 450 nm. Experiments were performed on n=3 independent occasions, with data expressed as pg/ml.

Isolated macrophage cultures ($5x10^5$ cells/ml) were established in RPMI-1640 medium with 10 % heat-inactivated FCS, in the absence and presence of sap (at sub-lethal concentrations of 0.01 %, 0.1 % and 1 %). Macrophages were subsequently stimulated to undergo polarisation into M1 macrophages, with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 ng/ml, PeproTech, London, UK; (Xu et al., 2015). Control cultures included unstimulated (GM-CSF-free) macrophages and macrophages with the highest concentration of sap (1 %), but without GM-CSF stimulation (to confirm the absence of sap pro-inflammatory effects). Cultures were maintained at 37 °C/5 % CO_2 for 7 days. M1 macrophage conditioned media were subsequently processed, with IL-6 and TNF-α levels quantified as described above. Experiments were performed on n=3 independent occasions, with data expressed as pg/ml.

2.8. Preliminary cytotoxicity assessments with dermal fibroblasts

Briefly, normal adult human dermal fibroblasts (Lonza, Basel, Switzerland) were cultured in BulletKit medium (Lonza) and seeded into 96-well plates at $5x10^3$ cells/well and maintained overnight at 37°C/5% CO_2. Media were subsequently replaced with BulletKit medium with serial dilutions of the aqueous sap (at concentrations of 0.01 %, 0.1 % and 1 %). Cultures were maintained at 37 °C/5 % CO_2 for 48 h, with fibroblast viability assessed using CellTiter Reagent (Promega, Southampton, UK) and reading absorbance at 490nm, as described above.

2.9. 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). Dermal fibroblast and HaCaT cultures ($2.5x10^3$ and $1x10^3$ cells/well, respectively), were seeded into 96-well plates in DMEM medium, supplemented with 1 % antibiotics/antimycotics (100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulphate and 0.25

μg/ml amphotericin B), 2 mM L-glutamine and 10 % foetal calf serum (all ThermoFisher Scientific); followed by incubation in serum-free DMEM for another 24 h. Serum-free media were subsequently replaced with 1 % serum-containing DMEM with sap (at sub-lethal concentrations of 0.01 %, 0.1 % and 1 %). Cultures were maintained at 37 °C/5 % CO_2 for 24 h or 72 h, with fibroblast and HaCaT proliferation assessed using the MTT (Sigma) dye-reduction assay (Mosmann, 1983). Absorbance values were quantified at 540 nm, as described above. Experiments were performed on n=3 independent occasions, with data expressed as a % versus untreated controls.

2.10. 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-Tukey test for multiple comparisons. Significance were considered at $p<0.05$.

3. Results

3.1. NMR spectra suggest the water soluble fraction of Lepiniopsis ternatensis sap contains the polyphenol trifucol

The ^{1}H NMR spectrum showed two resonances at 7.17 (d, J = 5.7 Hz, 1H) and 6.84 (m, 1H). The ^{13}C and DEPT NMR spectra showed 8 carbon resonances at 154.2, 154.1, 147.3, 128.6, and 128.4 (fully substituted), 131.7, 115.0 and 114.9 (trisubstituted), hence methine resonances. The HSQCDEPT spectrum showed that the ^{13}C NMR resonance at 131.7 corresponded to the proton resonance at 7.17, whereas the ^{13}C NMR resonances at 115.0 and 114.9 corresponded to overlapped proton resonance at 6.84. See supplementary section for spectra. As the ^{1}H and ^{13}C NMR data were too few for a meaningful structural elucidation from first principles, a spectral similarity search using the CSEARCH-Technology (Robien, 2019), was conducted for the 8 carbon resonances with the multiplicities provided and restricting elements present to C, H, O and N. The analysis of the spectral results showed a match with the known compound trifucol (Glombitza et al., 1975).

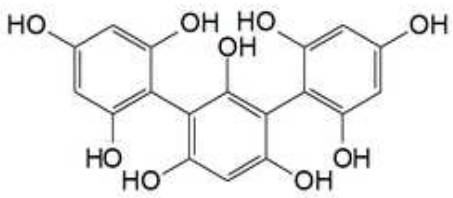

Fig. 1. NMR spectra from 1D and 2D experiments suggest the aqueous fraction of the sap contains trifucol as a single major component.

3.2. Lepiniopsis ternatensis sap is not antibacterial towards wound pathogens

The obvious starting point for evaluating a plant sap used to treat bacterial skin infections is to assess its antibacterial activity. A broth microdilution assay was carried out using the drug-sensitive strain *S. aureus* 6571 as the test organism with serial dilutions of the sap starting from 6% v/v. Despite the high test concentration used, no reduction in in growth compared to control was observed. Next, the sap was tested with 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; Aadrians and Shah, 1988; Noguera-Julian et al., 2019). The topical antiseptics chlorhexidine and povidone iodine were included as positive controls. 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 inhibitory activity towards the three pathogens. Positive controls displayed activity as expected.

	S. aureus	*S. pyogenes*	*F. ulcerans*
*Lepiniopsi*s 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) with *S. aureus, S. pyogenes* and *F. ulcerans* in a disc diffusion assay. Each 6mm paper disc was treated with 15 µl plant sap, 15µl 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).

3.3. The sap does not show signs of cytotoxicity towards dermal fibroblasts

To assess the potential cytotoxicity of the sap towards human skin cells, we initially determined its effects on normal adult dermal fibroblast viability using CellTiter Reagent. As Fig. 2a. shows, the sap exhibits no signs of cytotoxicity at concentrations up to 1 % v/v. This suggests that the sap is well-tolerated and provided a rationale for testing with other wound cell types.

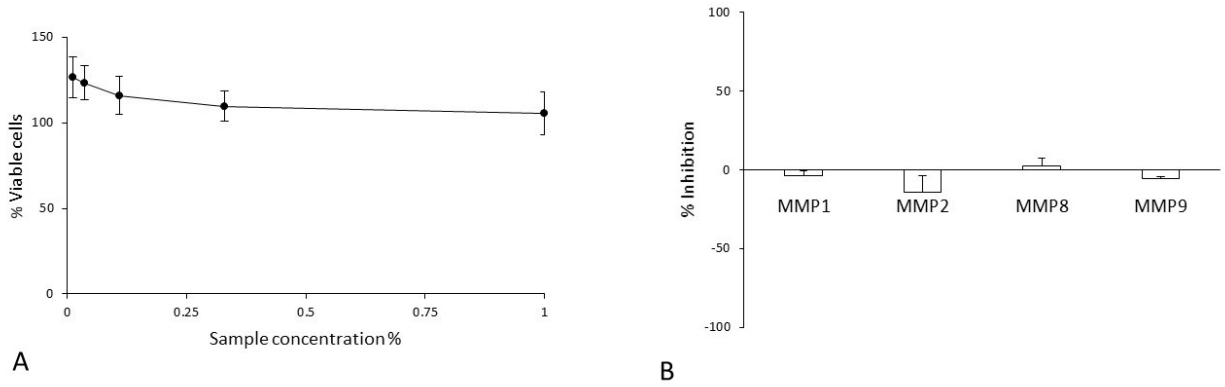

Fig. 2. Cell viability of normal adult dermal fibroblasts exposed to increasing concentrations of the sap over 48 h (A). Sap effects on the inhibition of MMP-1, -2, -8 and -9 activities, using a pro-fluorescent peptide substrate (B).

3.4. The sap does not inhibit wound MMP activities

MMPs, such as 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, wound applications that act to inhibit excessive MMP activities may help to promote wound healing. The sap was tested against recombinant MMP enzymes using a quenched fluorogenic peptide substrate. Preliminary experiments were undertaken to determine the highest sap concentration that did not quench fluorescence from the MMP reaction product (0.1 % v/v) and thereby, interfere with the assay. The sap was then tested with four different MMP enzymes. Fig. 2b. shows that the sap does not inhibit any of the four MMPs assessed.

3.5. The sap stimulates proliferation of dermal fibroblasts, but not keratinocytes

During normal wound healing, fibroblasts mediate numerous responses to enhance wound closure, such as the secretion of growth factors and deposition of extracellular matrix components, enabling wound re-epithelisation by keratinocytes (Moseley et al., 2004). 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. 3a and 3b show that although the keratinocytes show no significant increases in proliferation in response to the sap (all $p>0.05$), fibroblasts exhibit significantly increased proliferation at 72 h post-treatment ($p<0.01$ at 0.01 % sap concentrations; $p<0.05$ at 0.01 % and 1% concentrations), with the lowest concentration (0.01 % v/v) corresponding to a concentration of just 1.3 µg/ml soluble plant compounds.

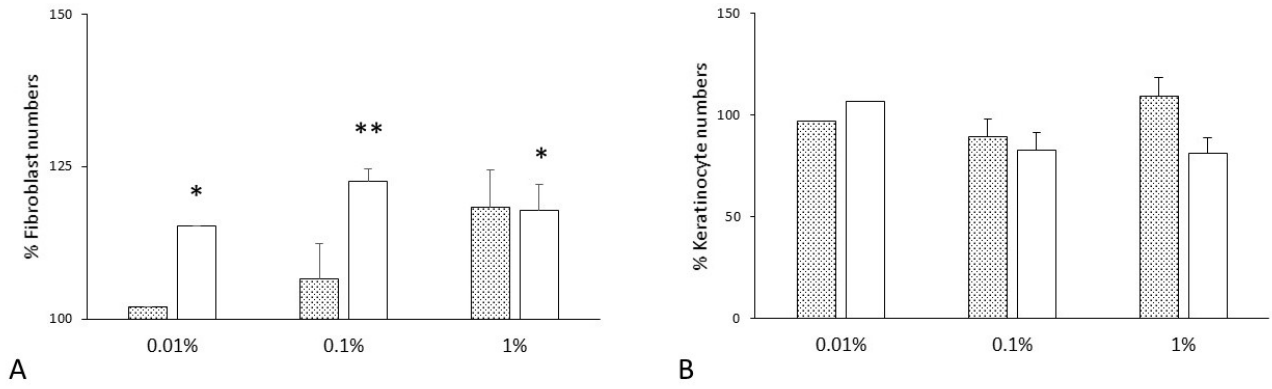

Fig. 3. Sap effects on dermal fibroblast proliferation at 24 h (shaded) and 72 h (unshaded), relative to untreated controls (3A). Sap effects on keratinocyte proliferation at 24 h (shaded) and 72 h (unshaded), relative to untreated controls (3B). Error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments. P values *$p<0.05$, **$p<0.01$.

3.6. High sap concentrations inhibit TNF-α release by M1 macrophages

We next wanted to understand the effects of the sap on the pro-inflammatory cells (neutrophils and M1 macrophages) that reside within chronic wound environments. It is well-established that perpetual inflammation in chronic wounds leads to increased expression and activities of MMPs, which in turn leads to extracellular matrix degradation, thereby disrupting healing and preventing would closure (Moseley et al., 2004; Barrientos et al., 2008). Therefore, we tested the sap to assess its influences on neutrophil responses, in addition to the polarisation and activation of pro-inflammatory M1 macrophages, via the quantification of changes in their secretion of pro-inflammatory cytokines, IL-6 and TNF-α. Rather than using secondary inflammatory cell lines, we used human-derived primary cells which more closely resemble the status of these cells *in vivo*. Controls with and without stimulation with LPS (neutrophils) or GM-CSF (macrophages) were also included to verify the stimulation of cells, as expected. Controls consisting of no LPS or GM-CSF stimulation, but with plant sap at 1 % (the highest test concentration), were included to determine if the sap acted to stimulate the cells by itself. Fig. 4a-4d show that for both neutrophils and M1 macrophages, the sap does not act to promote either IL-6 or TNF-α release, independently of LPS or GM-CSF stimulation. However, Fig. 4a shows that when M1 macrophages were stimulated with GM-CSF in the presence of the highest concentration of sap (1 % v/v or 130μg/ml), secretion of the pro-inflammatory cytokine, TNF-α, was significantly inhibited ($p<0.01$). Conversely, TNF-α downregulation was not observed with neutrophils (4C) and IL-6 release from neutrophils or M1 macrophages were unaffected at all sap concentrations assessed ($p>0.05$) (Fig. 4B and 4D).

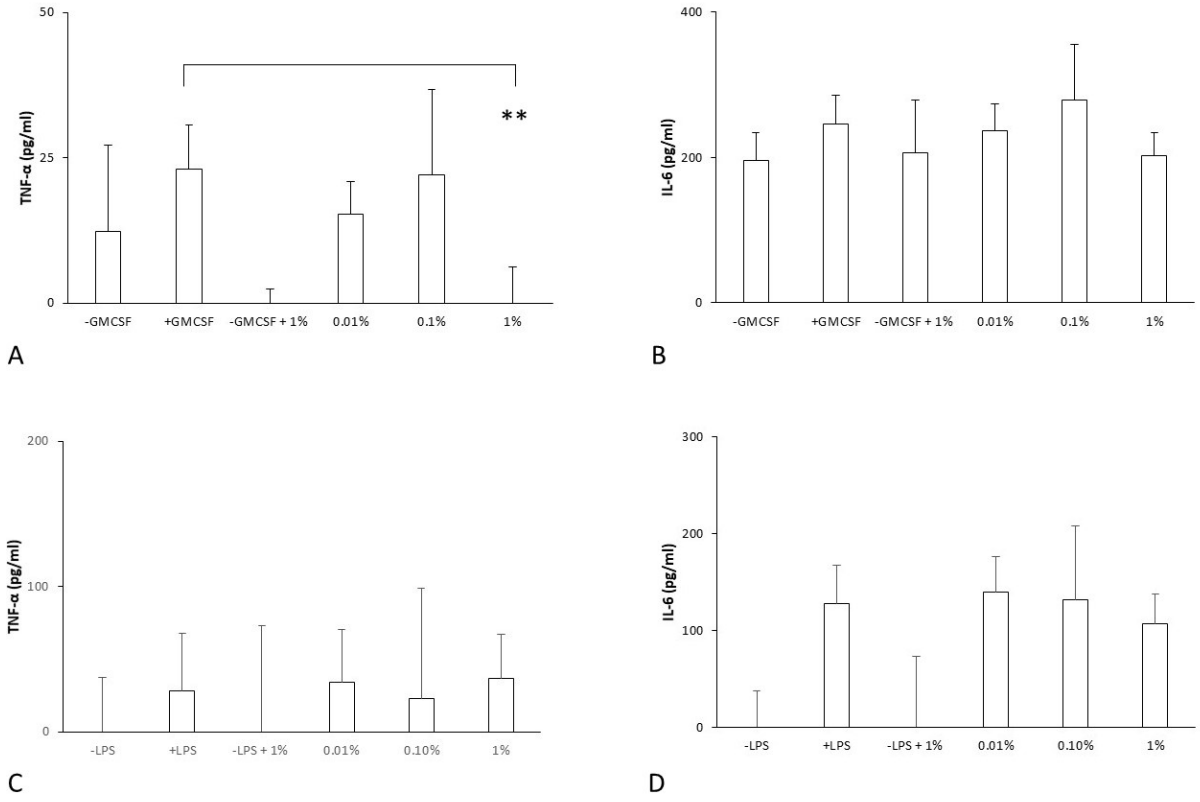

Fig. 4. Effect of the sap on TNF-α (A) and IL-6 (B) secretion by M1 macrophages. All conditions included stimulation with GM-CSF, unless otherwise indicated. % indicates % v/v of sap. Controls – GM-CSF and +GM-CSF indicate cell responses with and without GM-CSF, in the absence of sap. Control -GM-CSF + 1 % sap was included to check for sap-induced stimulation and uses cells with sap at 1 % v/v, but without GM-CSF stimulation. Bars marked 0.01 %, 0.1 % and 1 % are GM-CSF-stimulated cells with sap concentrations in % v/v, as indicated. Effect of the sap on TNF-α (C) and IL-6 (D) secretion by neutrophils. All conditions included stimulation with LPS, unless otherwise indicated. % indicates % v/v of sap. Controls -LPS and +LPS indicate cell responses with and without LPS, in the absence of sap. Control -LPS + 1 % sap was included to check for sap-induced stimulation and uses cells with sap at 1 % v/v, but without LPS stimulation. Bars marked 0.01 %, 0.1 % and 1 % are LPS-stimulated cells with sap concentrations in % v/v, as indicated. For all graphs, error bars indicate standard error of the mean (SEM) values, based on data analysis from n=3 independent experiments. P values *$p<0.05$, **$p<0.01$.

4. Discussion

The water soluble fraction of *Lepiniopsis ternatensis* sap contained a single major component which was identified though a spectral similarity search as the phlorotannin trifucol. Phlorotannins such as trifucol are thought to be marine-exclusive metabolites and have not to the best of our knowledge been

reported previously from higher plants (Catarino et al., 2017). Further work should be carried out with a greater range of sap samples from different *Lepiniopsis* species to confirm the distribution of the compound in the genus. Future work should also consider the possibility that biosynthesis of the compound is achieved via a fungal symbiont rather than from the tree itself.

The assay results presented here show the sap has potentially beneficial effect on wound healing. The sap significantly decreased M1 macrophage TNF-α secretion, a cytokine known to contribute to the non-healing state of chronic wounds, such as venous ulcers, diabetic ulcers and pressure sores; and thus seen as a potential therapeutic target (Moseley et al., 2004; Barrientos et al. 2008; Ashcroft et al. 2012). Inhibition of TNF-α release occurred at a sap concentration of 1 % v/v (130 µg/ml), which would be readily achievable near the wound surface, as the sap is applied topically to the wounds. Interestingly, neither TNF-α secretion from neutrophils, or IL-6 from neutrophils or M1 macrophages, were significantly reduced. This suggests that TNF-α macrophage-specific mechanisms may be involved. We intend to undertake further studies to elucidate the precise mechanisms responsible for these observations. Whether the suggested benefits of TNF-α inhibition would translate into therapeutic benefits in cutaneous ulcers in Papua New Guinea is uncertain at present, but the inflamed nature of these wounds implicates TNF-α in their pathogenesis, suggesting that this indeed may be the case.

The sap is also a potent stimulator of dermal fibroblasts, with stimulation occurring at just 1.3 µg/ml (0.01 % v/v). The effects were particularly seen at 72 h post-treatment, but only with fibroblasts and not keratinocytes. This suggests that the mechanism of stimulation involves fibroblast-specific cell signalling. Again, further work should be carried out to determine the precise mechanisms through which the effect is mediated. The relatively potent activity raises the possibility that small quantities of active compounds that diffuse deeper into the wound may be able to increase fibroblast proliferation *in vivo*, thereby contributing towards extracellular matrix deposition, growth factor production and other processes crucial for late stage wound healing. In non-healing chronic wounds, inflammatory cell-derived reactive oxygen species are believed to cause DNA damage in fibroblasts, thereby contributing to fibroblast senescence (Sindrilaru et al., 2011). Thus, by reducing M1 macrophage pro-inflammatory responses and stimulating fibroblasts, the sap may be able to address this defect via two independent mechanisms. Also, the physical attributes of the sap may impart a therapeutic benefit. By acting as a non-cytotoxic wound covering, the sap may prevent re-infection of healing wounds from pathogens in the external environment as well as preventing direct contact of flies that like to feed on wound exudate of these ulcers in Papua New Guinea.

Declaration of competing interests

The authors declare no conflict of interest.

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Figure 1: ^{1}H NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

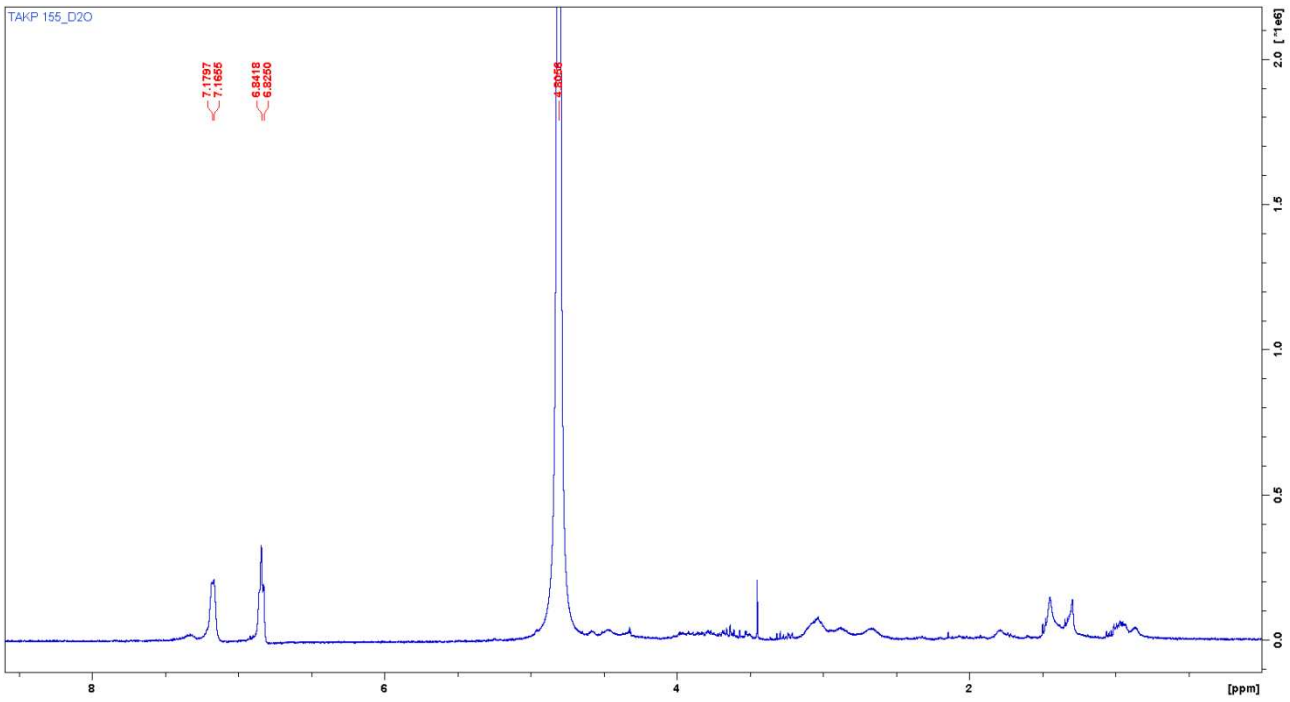


Figure 2: Expanded ^{1}H NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

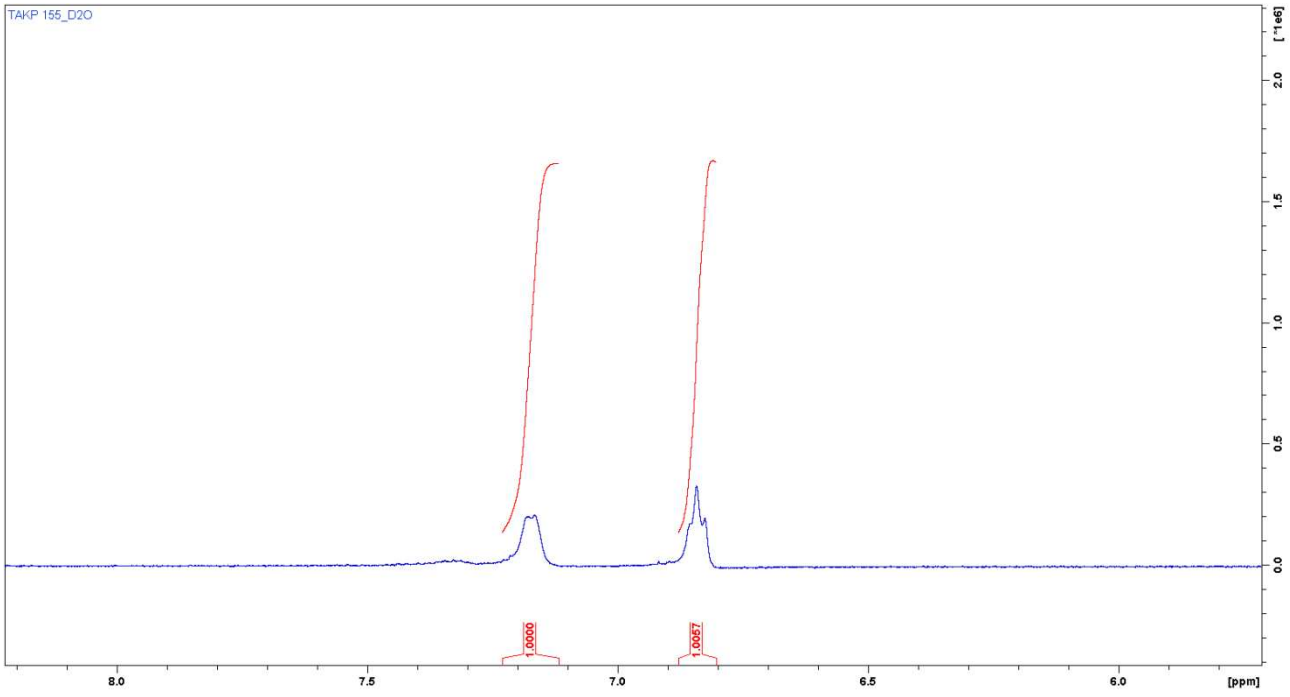


Figure 3: ^{13}C NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

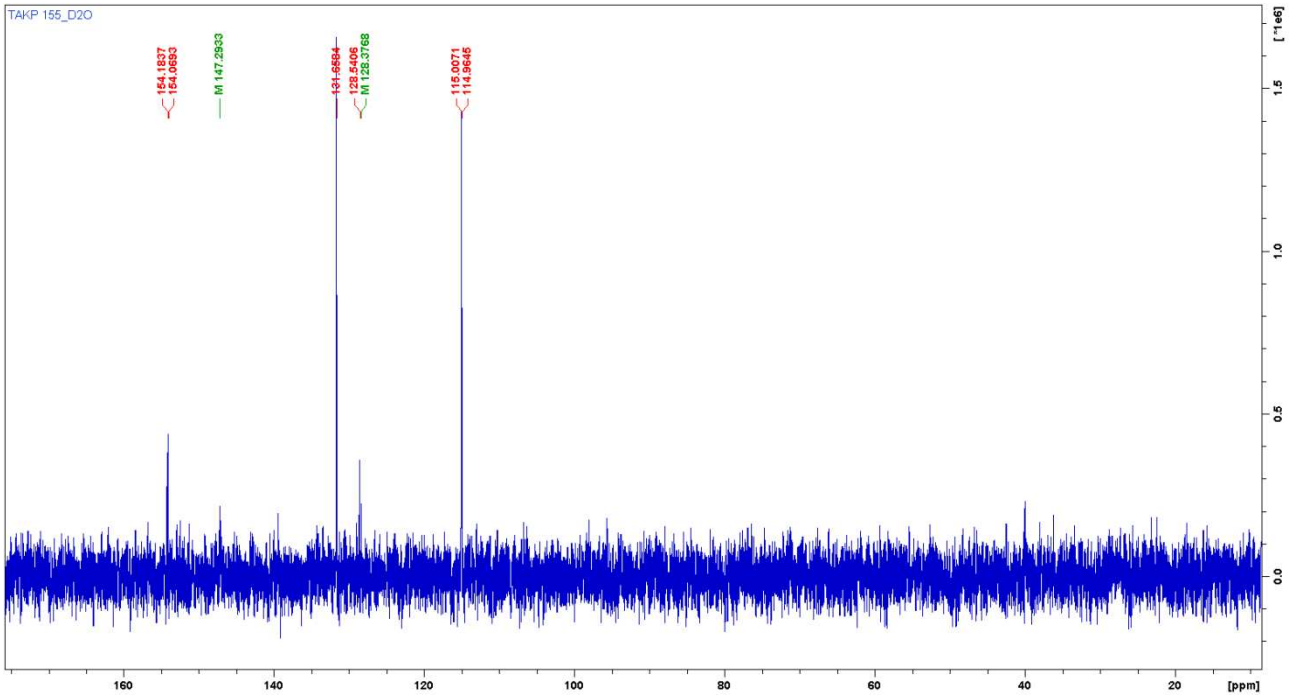


Figure 4: DEPT NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

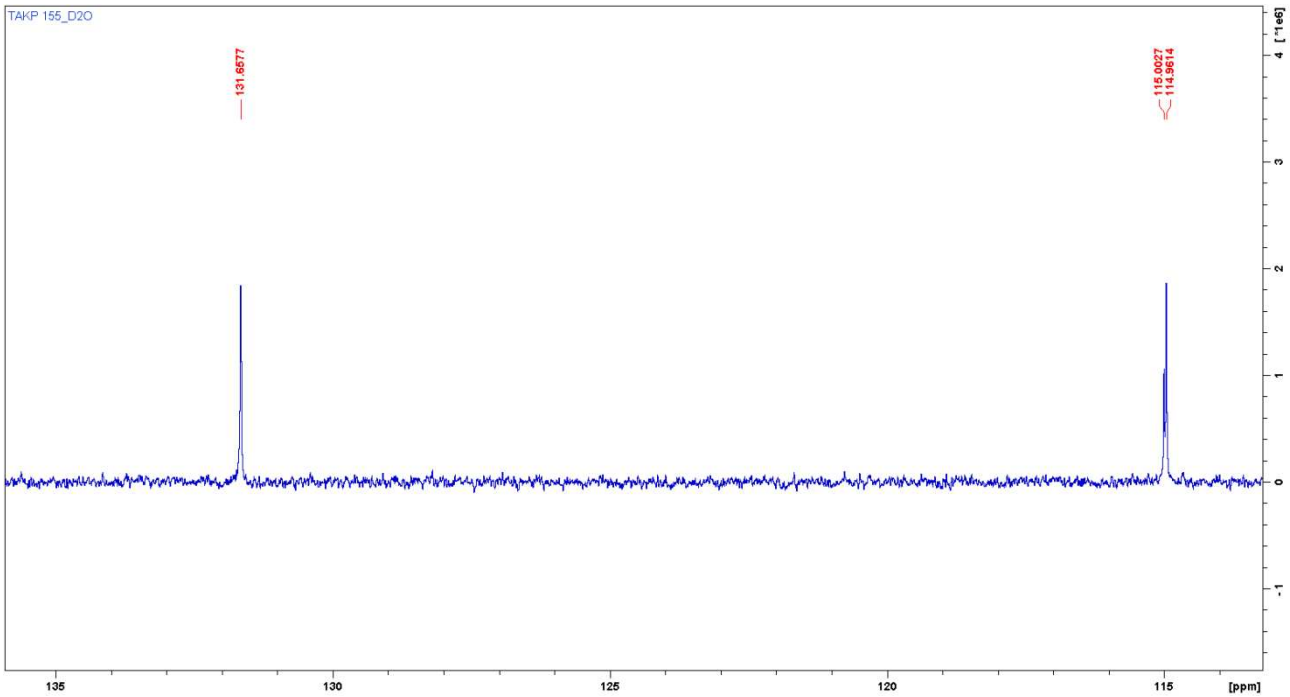


Figure 5: HSQCDEPT NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

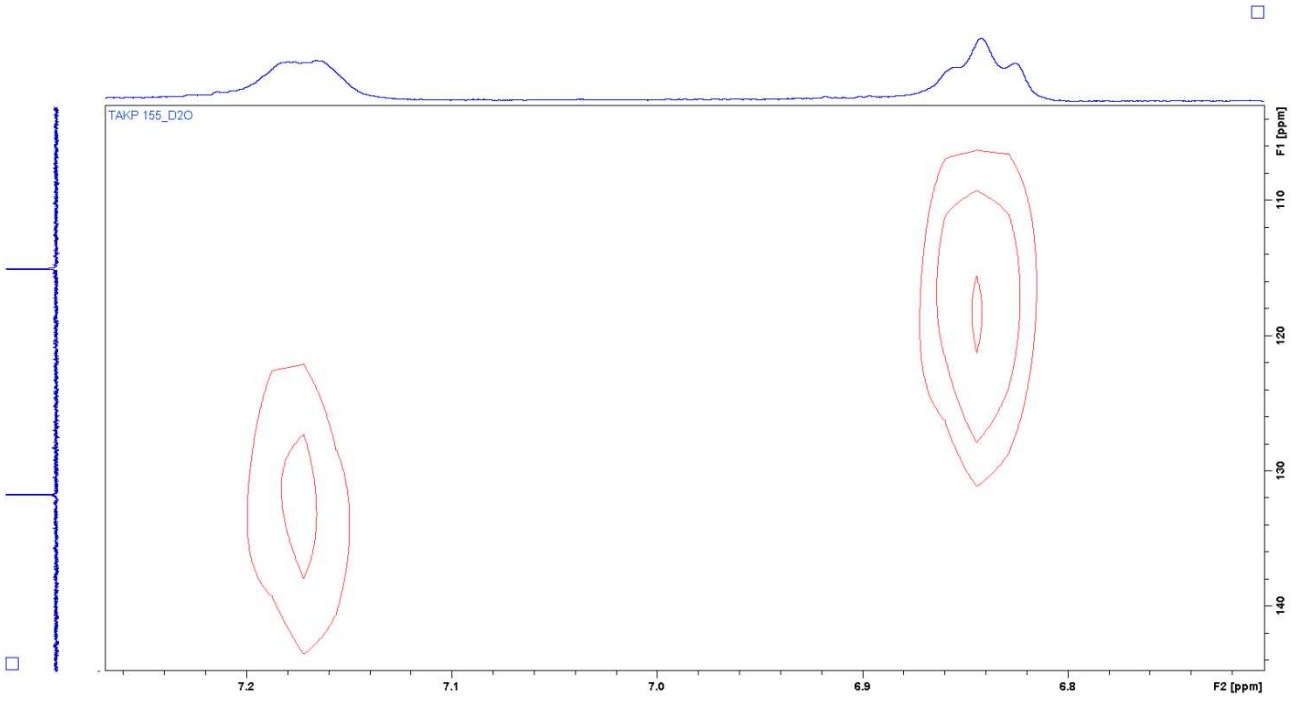


Figure 6: HMBC NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

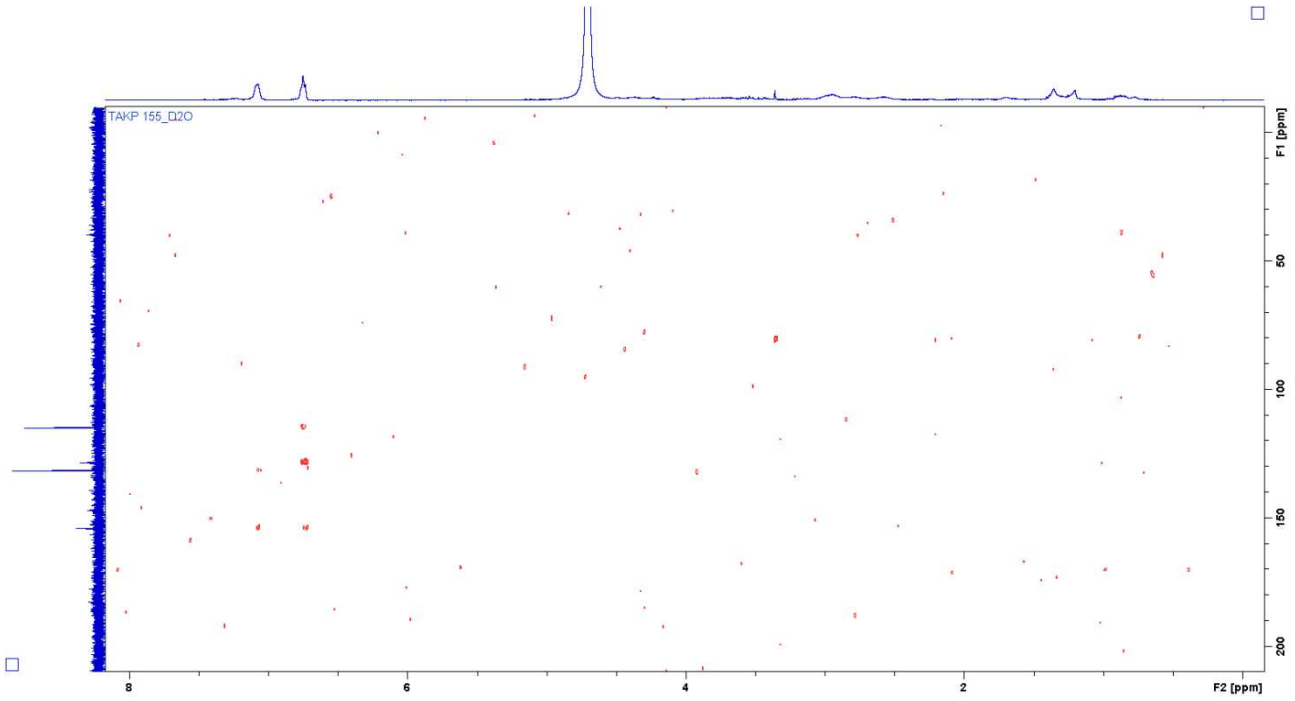


Figure 7: HMBC NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer

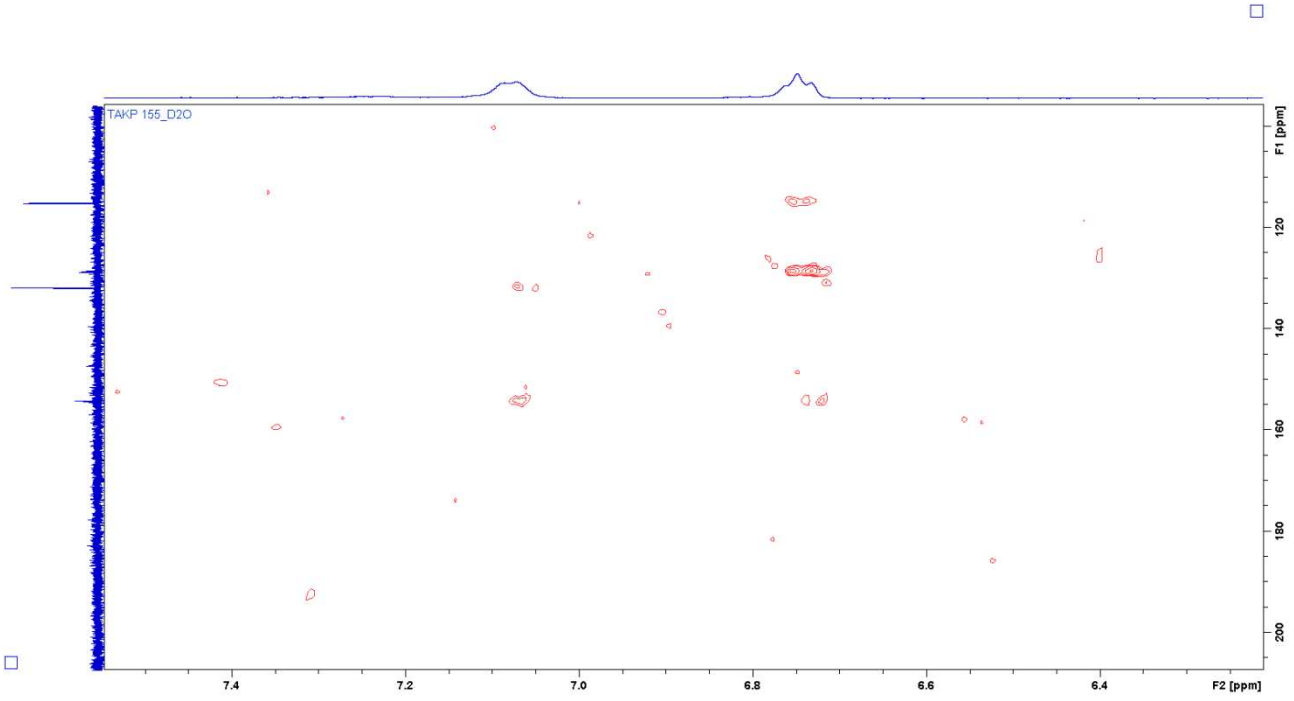
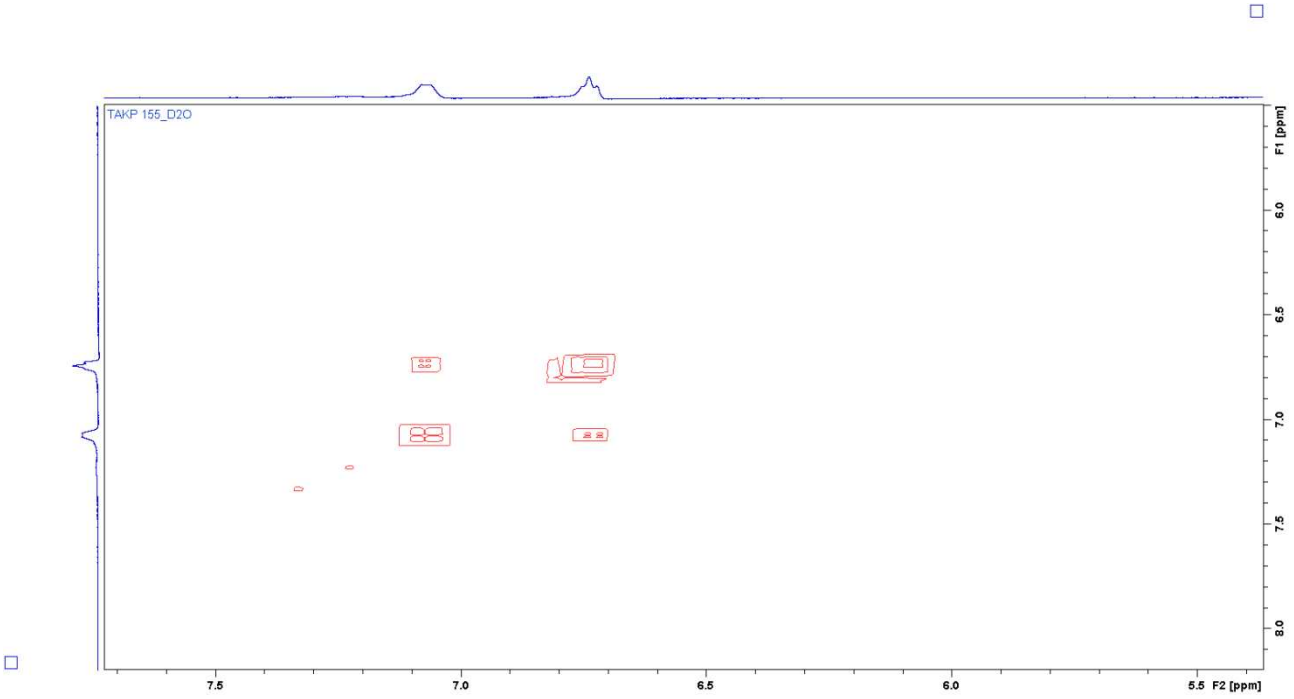


Figure 8: COSY NMR spectrum of lyophilised *Lepiniopsis ternatensis* sap analysed using D2O in a 400 MHz NMR spectrometer



Conflict of interest statement

Declarations of interest: none.

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