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Preparation of a Natural Product Extract Library for Investigation Against Disease States Specific to Defence Health: A Mini Long Range Research Project

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ABSTRACT

This technical report outlines the research activites conducted during a mini Long Range Research project to establish a natural product extract library for drug disocvery. The library was established with a view to it being screened against Defence health related targets. Discussed in this report are the research results generated from this project including establishing and chemically fingerprinting the extract library, *in vitro* results from screening a small selection of the library for antimalarial activity against parasites of *Plasmodium falciparum*, and the elucidation of the chemical structures of two compounds. The viability of conducting this type of research within the Human Protecton and Performance Division at DSTO is also discussed.

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Executive Summary

The Australian Defence Force (ADF) encounters a unique set of disease states that the greater Australian population is not necessarily exposed to on a routine basis. Consequently, from a Western medicine perspective, the ADF has a specific requirement for therapeutics to treat disease states that are not part of the standard research programs of mainstream pharmaceutical / biotechnology sector (examples include but are not limited to vector-borne diseases and melioidosis). In particular, the threats posed from vector-borne diseases are constant to deployed forces in many countries, and require specific and strict prophylaxis regimes.

During the 2007 / 08 financial year a mini Long Range Research (mLRR) project was conducted looking to establish a natural product-based extract library for screening against relevant Defence health diseases. In total 131 plant and marine specimens were collected from Victoria. These specimens were extracted to create the extract library, and chemically fingerprinted using various analytical techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS) and proton (^{1}H) Nuclear Magnetic Resonance (NMR) spectroscopy. During the mLRR a Summer Vacation Student (SVS) aided in the preparation of extracts, and also undertook chemical investigations in an extract of a *Grevillea* sp. These investigations led to the isolation of two compounds from an extract of a specimen of *Grevillea* collected from the Fishermans Bend site. Fortuitously one of the isolated compounds was found to be a novel compound. Finally, seven plant extracts were selected for *in vitro* antimalarial screening against parasites of *Plasmodium falciparum*, in conjunction with the Army Malaria Institute. Of the extracts sent for screening, four were shown to have activity against malaria, with one extract (from a *Grevillea* sp.) having quite strong activity.

Discussed in this technical report are the merits of conducting this type of research within DSTO, the results obtained from the mLRR project, the conclusions that can be drawn from it and broader capability gains that can be made from conducting this research.

Authors

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Simon Ovenden graduated with a BEd-Sci in 1994 and with a BSc(Hons) in 1995 from The University of Melbourne. In 1999 he completed a PhD in marine natural products chemistry from the same institution. He then completed two years post doctoral research in Singapore at the Centre for Natural Product Research isolating and elucidating novel natural products as potential drug leads. Following this Simon spent three years at Cerylid Bioscience in Melbourne, then approximately one year at the Australian Institute of Marine Science, in both cases as a Senior Research Scientist researching novel natural products as potential drug leads from Australian biota. He joined DSTO in 2006 as a Defence Scientist in Analysis and Verification, where he is applying his background in Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry.

Marina Chavchich
Army Malaria Institute

Marina Chavchich graduated from the Polytechnical Institute in Saint-Petersburg (Russia) with MSc degree in Biophysics in 1988. She completed her postgraduate training in Biochemistry at the Roentgen Radiology Institute in Saint-Petersburg and spent next three years working as a Research Officer at Genetic Engineering Laboratory at the same Institute. In 1997, she immigrated to Australia and worked as a Research Assistant in the Malaria Laboratory at the Queensland Institute of Medical Research. In 2000 she received the University of Queensland Graduate School Award and enrolled into a PhD program on comparative genomics of the two human malaria parasites Plasmodium falciparum and P. vivax. In 2003 she joined the WRAIR laboratory at the Australian Army Malaria Institute, where she worked on mechanisms of artemisinin resistance in P. falciparum. Marina completed her PhD in 2005 and joined the Department of Drug Evaluation as a Research Scientist, where she is applying the molecular and in vitro techniques for evaluation of new antimalarial compounds as well as providing support to clinical trials of antimalarial drugs.

Melanie Cobbe
Human Protection and Performance Division

Melanie is currently studying a double degree in Science and Commerce at The University of Melbourne. During the summer of 2007/08 she received a Summer Vacation Scholarship to spend 12 weeks in the Human Protection and Performance Division conducting research into natural products.

Eloise J. Pigott
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Eloise Pigott graduated with Honours in a Forensic Science degree at Deakin University (Waurn Ponds) in 2006. She joined DSTO in 2007 where she works as a Defence Scientist in Analysis and Verification and is involved in helping to establish a strong forensic capability in the Human Protection and Performance Division.

Melissa J. Laws
Human Protection and Performance Division

Melissa Laws graduated with a BSc(Hons) in 1994 from the University of Melbourne. In 1998 she completed a PhD in organic chemistry, developing new free-radical syntheses of some organoselenides and organotellurides at the same institution. Melissa then undertook a year's postdoctoral research with Professor Samir Zard at L'Institut de Chimie des Substances Naturelles, Le Centre National de la Recherche Scientifique, Gif-sur-Yvette in France. On her return to Australia, Melissa began work in the area of medicinal chemistry, developing partial agonists of adenosine A1 receptors; firstly at Deakin University and then Monash Univeristy, during three years' postdoctoral research with Professor Peter Scammells. Melissa also has significant experience in the chemical industry having worked at IDT Australia and as a senior scientist at Chirogen Pty Ltd. She joined DSTO in 2005 as a Defence Scientist and divides her time between work in the synthesis group and arms control and counter-proliferation efforts.

Michael D. Edstein
Army Malaria Institute

Michael Edstein graduated in 1975 with a BSc degree from the Australian National University, majoring in Biochemistry. He joined the Australian Army Malaria Research Unit (now known as the Army Malaria Institute - AMI) in 1975, where he was responsible for developing HPLC methods for the measurement of antimalarial drugs in biological fluids. He completed his MSc in 1985 and his PhD in 1995 at the Faculty of Medicine, University of Sydney. During and following his postgraduate training, his primary focus has been the preclinical and clinical evaluation of the pharmacokinetics and pharmacodynamics of new antimalarial drugs for the treatment and prophylaxis of ADF personnel against malaria infections. He was the Commanding Officer of AMI from 1996 to 2002. His current appointment is Head, Department of Drug Evaluation at AMI.

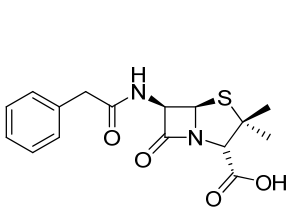
Contents

Glossary

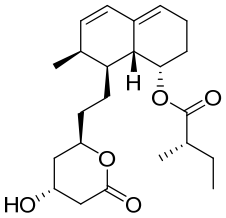
ACN	Acetonitrile
AMI	Army Malaria Institute
COSY	Correlation Spectroscopy
DMSO	Dimethyl Sulfoxide
EtOH	Ethanol
FTMS	Fourier Transform Mass Spectrometry
gHMBC	gradient Heteronuclear Multiple Bond Correlation spectroscopy
gHMQC	gradient Heteronuclear Multiple Quantum Coherence spectroscopy
HPLC	High Pressure Liquid Chromatography
IC_{50}	Inhibition at 50% concentration
LC-MS	Liquid Chromatography-Mass Spectrometry
MeOH	Methanol
mLRR	mini Long Range Research project
NMR	Nuclear Magnetic Resonance spectroscopy

1. Introduction

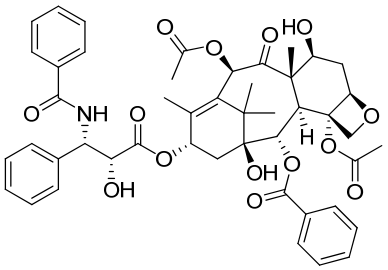
Over the lifetime of the modern pharmaceutical industry, natural products have been established as an excellent supply source for the discovery of novel compounds with therapeutic potential.[1-4] Compounds such as the antibiotic penicillin G (**1**), and lipid lowering compounds belonging to the statins structure class (such as mevastatin (**2**), isolated from the mould *Penicillium citrinum*) have demonstrated the significant role natural products have played to deliver novel therapeutics. More recently, the anticancer compound Taxol™ (**3**), isolated from the Pacific yew tree, has been a "blockbuster" for Bristol Meyers Squibb, with sales in excess of US$1.6 billion in 2000.[5] Recent literature has shown that nature is more adept at producing compounds that are chemically more diverse than humans are capable of producing at the bench.[6] Indeed, despite the other techniques (e.g. combinatorial chemistry and rational drug design) now applied to the endeavours of drug discovery, natural products continue to have significant influence in the drug discovery industry. From 1981 until June 2006, of the 974 small molecule drugs released to the market, 51% were natural products based, or derived from core structural motifs that were based on natural products.[4]



penicillin G (**1**)



mevastatin (**2**)



Taxol™ (**3**)

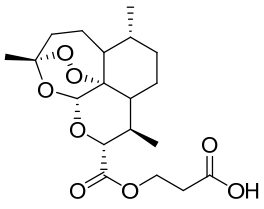
The Human Protection and Performance Division (HPPD) within DSTO is uniquely placed to conduct research in the field of Defence health, and in particular vector-borne disease and more specifically malaria. There is significant industry expertise in natural products drug discovery, with complementary industry experience in medicinal chemistry. Furthermore Australia is one of seven mega diverse countries in the world and as such has a unique biota on which to draw for samples. There are clear requirements from a client base (the ADF through the Defence Support Group or DSG and, in particular, Defence Health Services or DHS) for the discovery of novel treatments to Defence specific diseases. Defence has a renowned research institute that is specifically focussed on conducting research into vector-borne diseases, namely the Army Malaria Institute (AMI). The combination of the above unique circumstances grants HPPD significant opportunities to further explore the feasibility of conducting natural products research with a view to specific ADF disease targets.

Considering the above assessment, a mini Long Range Research (mLRR) project proposal was accepted that looked at establishing a natural product extract library to screen against an antibacterial target. Due to unforeseen circumstances the focus was changed to establishing an extract library for DSTO to exploit in screening against relevant ADF disease targets. The mLRR research project was designed to try and meet the following broad objectives:

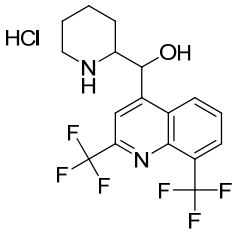
- To collect and process specimens of terrestrial plants and marine invertebrates from Victoria;
- To prepare an extract library from these specimens for screening against a relevant Defence health related biological target; and
- To conduct LC-MS and NMR profiling of these extracts to help aid in dereplication.

Following a visit by Prof Dennis Shanks (Director, AMI) and subsequent follow up discussions, an additional objective of conducting a pilot screening program of several extracts *in vitro* against *Plasmodium falciparum* isolates was also added.

Malaria continues to be one of the most debilitating and devastating diseases to humans. Each year 350-500 million malaria cases occur worldwide with more than 1 million people dying from falciparium malaria.[7] In an effort to delay the spread of multiple drug-resistant parasites the World Health Organization has recommended that artemisinin-based combination treatments (ACT) become the "policy standard" for the treatment of falciparum malaria.[8] The combination of a highly potent artemisinin compound to rapidly kill parasites with another structurally unrelated and more slowly eliminated partner drug that persists sufficiently long enough in the blood to prevent recurrences of malaria are now considered the best therapy for the treatment of *P. falciparum* malaria.[9] However, there is genuine concern that drug resistance against one of the most efficacious ACTs, artesunate-mefloquine (**4** and **5**)[10] is starting to appear on the Thai-Cambodian border. While malaria vaccine development has undertaken significant strides in the last few years, a viable vaccine is still many years away with significant hurdles still to negotiate. Thus, there is still a significant requirement worldwide for the discovery of novel structure classes of small molecule therapeutics that have a different mode of action to ACTs.



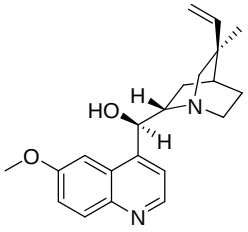
artesunate (**4**)



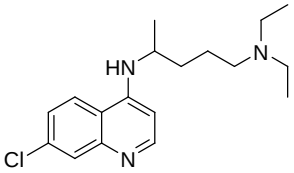
mefloquine (**5**)

In addition to the current history outlined above, malaria has also been responsible for jeopardising military campaigns. It had a major impact on Australian service personnel serving in the Middle East, western Pacific and Indo China theatres in World War II, the Korean War and the Vietnam War. More recently, the Australian Defence Force (ADF) experienced over 550 cases of malaria in personnel deployed on peacekeeping missions to Bougainville, East Timor and the Solomon Islands.[11] As an additional risk, ADF personnel may develop malaria after leaving malarious areas due to numerous factors such as inadequate prophylaxis, poor prophylactic compliance or drug resistant parasites. The current prophylaxis regime of insect repellents, antimalarial drugs and bed nets do not guarantee protection. Furthermore, once a person has contracted malaria, it remains with the patient and becomes an on going health concern with the person able to relapse at any time. Therefore the development of better antimalarial drugs to prevent and treat malaria is an ADF priority.

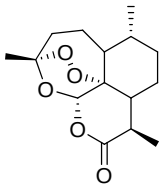
Drugs derived from natural sources have been the corner stone for treating people infected with malaria. Initially quinine (**6**), isolated from the bark of *Cinchona succirubra*, was the first natural product to show efficacy against malaria.[12] Subsequently, **6** has been the molecular basis for many other derivatives with antimalarial activity (such as chloroquine (**7**) and **5** above). Many years later artemisinin (**8**), isolated from the Chinese herb *Artemisia annua*[12], was found to be a potent inhibitor of malaria. Current front-line treatments for malaria are still either the natural products themselves, such as **8**, or semi-synthetic compounds based on existing natural products, such as **4** and **5** above.



quinine (**6**)



chloroquinine (**7**)



artimesinin (**8**)

This report will document the progress made in the mLRR towards the highlighted objectives above. It documents two pure compounds that were isolated from a *Grevillea* sp. collected from the Fishermans Bend site of DSTO and includes a discussion on the process involved in the elucidation of the structures. It will also make recommendations as to the viability of engaging in this area of research, the therapeutic areas that could benefit from this research, and the broader capability enhancements that can be achieved through conducting this research at HPPD.

2. Results and Discussion

2.1 Generation of extract library

It has been well documented that Australia has a unique and diverse terrestrial and marine biota, with Australia estimated to have around 10% of the worlds biota.[13] Unsurprisingly, from a natural products drug discovery stand point, most of this biota has yet to be investigated – indeed a significant amount has yet to be described.[13] This positions Australia as a rich biota source pool for this process. Additionally, due to the nutrient loaded temperate waters of southern Australia, there is a great diversity and a plentiful supply of invertebrate marine biota. Hence this pool of marine biological diversity is an excellent source for novel biologically active natural products.[14]

As a starting point for the creation of an extract library it was decided to initially focus on biota collected from sites within Victoria. This was due to the relative ease of collection and, more specifically, the diversity of marine invertebrate biota that is available. Shown in Figure 1 are the approximate locations of sites where collections have been made up to 30 June 2008. Currently, 69 plant and 62 marine specimens have been collected. Plant samples were collected from three sites. A significant majority were collected from the DSTO site at Fishermans Bend, with small collections made at Halls Gap and Mildura. Simultaneously,

marine invertebrates were collected from four sites, two inside Port Phillip Bay at Sandringham and Cerbrus, and two outside the heads near Cape Schanck.

Figure 1: Collection sites for marine and plant biota from around Victoria, with an expanded view of collections sites in Melbourne. Plant locations are shown with green arrows; marine locations are shown with blue.

Once biota is collected, it needs to be processed in such a way as to create an extract for both biological testing and for chemical fingerprinting. A flow diagram outlining the procedure followed for the extract library is shown in Figure 2. All available plant and marine specimens (131 in total) were processed in this manner. Depending on the type of biota collected (marine or terrestrial), the extract may be generated using one of two subtly different processes.

For plant extracts, the collected biota is freeze dried to remove all H_2O (step a). Once dried, the specimen is ground to a powder (step b) from which approximately 2 g is taken. This material is steeped in MeOH (approximately 10 mL) for a period of approximately 12 h (step c). The volume of MeOH is removed and the process repeated. This process removes the potentially interesting "extractable" intra- and extra-cellular compounds into the MeOH. The MeOH volumes are combined (≈ 20 mL), reduced in volume to approximately 3 mL and then treated with polyamide (step e). Polyamide is a solid phase sorbant that works by hydrogen bonding the polyphenolic compounds (such as tannins), which are interference compounds in certain biological assays and lead to false positive results. The polyamide treated extract is concentrated to dryness under a stream of N_2. The extract is then ready for chemical fingerprinting (LC-MS and ^{1}H NMR) and for biological assays (step f).

For the generation of marine invertebrate extracts, two small portions of each specimen are cut off. One portion is placed in a jar of EtOH as a museum type sample, while the other portion is freeze dried (step a). The freeze dried portion is then steeped overnight twice in MeOH (step d). The MeOH extracts are combined and concentrated to dryness. They are then resuspended in ≈ 5 mL of MeOH and filtered through tissue paper to remove the insoluble salt and residual cellular material (step e). The marine invertebrate extract is then concentrated to dryness under a stream of N_2. The extract is then ready for chemical fingerprinting (LC-MS and ^{1}H NMR) and for biological assays (step f). Marine extracts do not require pre-treatment with polyamide as they do not contain the polyphenolic compounds that plants do.

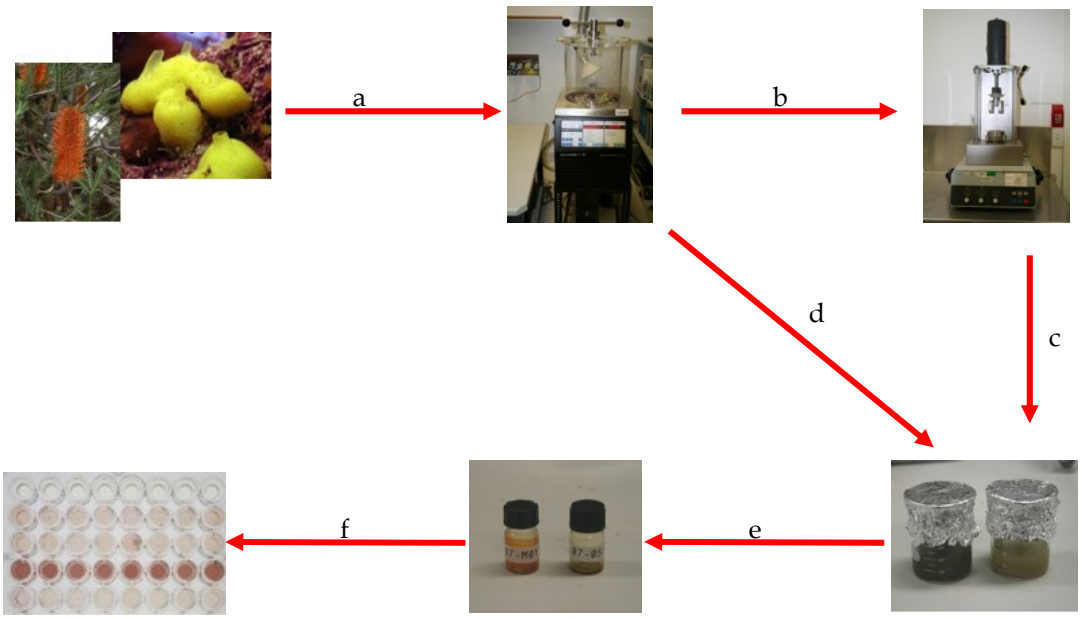


Figure 2: Schematic diagram highlighting each stage of the extraction process. a) Biota is collected and processed, with small samples taken from the collected specimen for preparation of the extract library. These samples are freeze dried; b) Plant samples are ground to a powder using a grinder; c) Dried plant material is extracted with MeOH; d) Post freeze drying the marine samples are directly extracted with MeOH; e) Final treatment step. Plant extracts are treated with polyamide to remove polyphenolic interference compounds. Marine extracts are filtered to remove the residual salt that falls out of solution; f) Extracts are then able to be submitted for biological testing.

2.2 Chemical Fingerprinting

Chemical fingerprinting of the generated crude extracts is important for several reasons. Initially it serves as an indicator of the complexity of the extracts and potentially allows for early identification of the types of chemistry (or chemical classes) that are present in the extracts. It also aids in the "dereplication" of crude extracts. The aim of dereplication is to make sure that replicate extracts are identified. Broadly, once a series of extracts have been identified to have bioactivity against the specific biological target of interest, one needs to make sure that each of those extracts is chemically unique. That is, the chemistry within the extract that is responsible for the biological activity is not replicated in other extracts. To allow for this discrimination to be made, a "chemical fingerprint" of the extract is made utilising LC-MS and ^{1}H NMR (see Appendix A for representative spectra). This information, coupled with bioactivity data, allows for efficient dereplication to occur. All 131 available specimens were fingerprinted using LC-MS and ^{1}H NMR (in d_4-MeOH), printed and filed for further reference as required.

2.3 Screening at the AMI

In collaboration with AMI, it was agreed to screen a small sub-set of the assembled natural product extract library for antimalaria activity. Subsequently, seven plant extracts were sent to the AMI for evaluation against the D6 *P. falciparum* cell line (see Table 1 for a list of the seven extracts sent for testing). The plant samples selected for testing were chosen with a view to covering extracts made from as many parts as possible that could be screened. Of all the extracts generated from the available plant parts, only extracts from bark were not screened, as at the time no extracts were available. Additional to this, a series of solubility studies were conducted to ascertain if the extracts could be solubilised in the screening buffer. Discussed in this section are these solubility studies, a description of the assay conditions for screening against *P. falciparum* lines and the results generated from this screening.

Table 1: Summary of extracts of plant material send to AMI for screening against the D6 line of P. falciparum

Extract number	Genus species	Plant part
07-008	*Persoonia picifolia*	Leaf and twig
07-010	*Grevillea sp.*	Leaf and twig
07-012	*Agonis flexuosa*	Stem
07-019	*Grevillea sp.*	Inflorescence and stem
07-020	*Acacia sp.*	Fruit
07-021	*Acacia sp.*	Inflorescence
07-027	*Agonis flexuosa*	Leaf and twig

2.3.1 Solubility studies

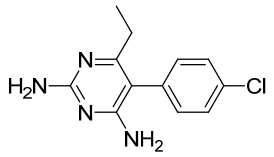
Traditionally, samples for screening are dissolved in aqueous organic solvent before screening is conducted. This is to make sure that as great a spread of polarity of compounds as possible are in solution. For the antimalarial screening conducted at AMI there was a requirement that there be no more than 0.25% of any organic solvent present in any of the screening wells. Hence studies were conducted to determine the solubility of these extracts at a maximum screening concentration of 500 μg/mL.

To this end 4 mg of each of three plant extracts were dissolved in 20 μL of organic solvent (either one of MeOH or DMSO). Of this a 2.5 μL aliquot was taken and made up to 100 μL with H_2O, giving a concentration of 2.5% organic. From this a 1:10 dilution was made (10 μL plus 90 μL of H_2O). This gave a final aliquot of 100 μL of 500 μg/mL in 0.25% organic solution.

Extracts were then studied for sediment through centrifugation, with no sediment detected. This method was then passed onto the AMI as a procedure for dissolving extracts for screening in aqueous media that satisfied the requirement that no more than 0.25% of the total assay volume be an organic solvent.

2.3.2 Antimalarial biological screening

The D6 *P. falciparum* parasite laboratory line originated from Sierra-Leone and is sensitive to most antimalarial drugs including chloroquine (**4**) and pyrimethamine (**9**), and was the line selected for the evaluation of antimalarial activity of these plant extracts. Two methods were used to evaluate the *in vitro* antimalarial activity of the plant extracts from DSTO: inhibition of ^{3}H-hypoxanthine uptake using 48 h and 96 h exposure[17] and microscopic analysis after 48 h exposure.



pyrimethamine (**9**)

The plant extracts described in Table 1 were dissolved in DMSO to the concentration of 0.1 mg/µL. Three 10-fold dilutions of stock solution were made using hypoxanthine-free complete media containing 10% human plasma to final concentrations in the well of 500 µg/mL, 50 µg/mL and 5 µg/mL. Aliquots (50 µL) of extract dilutions were added to 96-well microtitre plates and mixed with inoculum (50 µL) containing ring stages (6-12 h post invasion) of D6 parasites. Solvent blanks were tested alongside plant extracts to rule out inhibitory effect from the solvent.

The plates were incubated at 37 °C for ~24 h, then 20 µL of ^{3}H-hypoxanthine added and the plates re-incubated for a further 24 h or 72 h. Inhibition of parasite growth measured by hypoxanthine uptake assay[17] for the plant extracts was then determined. Additionally, ten 2-fold serial dilutions of the extracts with concentrations starting from 250 µg/mL to 0.244 µg/mL using the same *in vitro* conditions as described above were used to determine IC_{50} values for the plant extracts. Chloroquine was used as a reference drug.

For microscopic analysis, thin blood smears from the wells containing the highest concentration of the plant extracts were made to rule out the toxic effect of the extracts on red blood cell membrane integrity.

2.3.3 Results of screening

Shown below in Table 2 is the growth inhibition data at 48 h and 96 h for the seven plant extracts that were sent to the AMI for antimalarial testing. When assessing the activity of any extract of a natural product, it is important to bear in mind that each extract will contain upwards of 1000 different types of compounds. Hence the activity that is recorded for these initial crude MeOH extracts will be greatly reduced compared to the final activity of the compound(s) that are responsible for the activity. For the seven extracts initially tested, four recorded reasonably significant activity (>50% inhibition at 50 µg/mL), namely 07-010, 07-012, 07-019 and 07-027 (highlighted in yellow in Table 2). The activity recorded for 07-010 was particularly strong, with 100% growth inhibition still recorded after the first 1:100 dilution.

Table 2: In vitro antimalarial activity of plant extracts from DSTO against the D6 line of P. falciparum

Extract number	Parasite growth inhibition (%)					
	48 h assay Concentration in well (μg/mL)			96 h assay Concentration in well (μg/mL)		
	500	50	5	500	50	5
07-008	100%	24%	11%	100%	20%	5%
07-010	100%	100%	25%	100%	100%	36%
07-012	100%	54%	19%	100%	55%	2%
07-019	100%	94%	3%	100%	93%	2%
07-020	95%	11%	1%	96%	15%	0%
07-021	96%	24%	14%	98%	17%	7%
07-027	100%	82%	13%	100%	86%	8%
DMSO	3%	4%	1%	0%	0%	0%

Note: Shaded rows are extracts with significant activity

It was interesting to note that for all four plant extracts that had significant parasite growth inhibition after 48 h, the growth inhibitory properties were still maintained after 96 h. Microscopic examination did not show any effects on the erythrocyte membranes, with red blood cells appearing intact at the highest extract concentration tested (500 μg/mL). The three most active extracts appeared to affect the development of rings into trophozoite stage. In the wells containing 07-010 at 50 μg/mL, the D6 parasites did not progress further than early trophozoite stage. Shown in Table 3 are the IC_{50} values for the seven plant extracts, with the four most active extracts highlighted.

Table 3: IC_{50} values (μg/mL) for the seven plant extracts sent for antimalaria evaluation. The most active plant extracts are highlighted in yellow.

Plant extracts IC_{50} (μg/mL)							
	07-008	07-010	07-012	07-019	07-020	07-021	07-027
IC_{50}	166	6.4	34	33	102	>1000	48
IC_{50} 95% CI	88 to 312	5.8 to 7.1	31 to 37	32 to 33	89 to 116	NA	44 to 53

Again it is worth highlighting the excellent activity recorded for 07-010, with an IC_{50} = 6.4 μg/mL. As a comparison, in conjunction with the plant crude extracts the standard antimalarial drug chloroquine (**7**) was also screened, with an IC_{50} = 5.3 ± 0.3 ng/mL. While **7** is three orders of magnitude more active than 07-010, it is worth repeating that 07-010 is a crude extract, with upward of 1,000 compounds contained within the extract. This inherent matrix complexity will dilute the recorded activity of the compound responsible for the antimalaria activity of the crude extract.

Shown in Figure 3 are the dose response curves recorded for the seven extracts and the DMSO control. Interestingly, there is quite a difference in the slope of the dose response curves for the four active extracts. For 07-012 and 07-027, the slope is significantly shallower than those for 07-010 and 07-019. The extracts 07-012 and 07-027 were generated from different parts of the same specimen of *A. flexuosa*, while 07-010 and 07-019 were generated from different parts of the same specimen of *Grevillea* sp. (Table 1). At this stage it is unclear what is causing this difference in slope. It may be due in part to a different mode of action of the active

compounds in one set of plant extracts versus the other. It may also be a measure of reversible (shallow slope) versus irreversible (steep slope) binding of the active compounds. More work will be conducted studying this phenomenon if/when required.

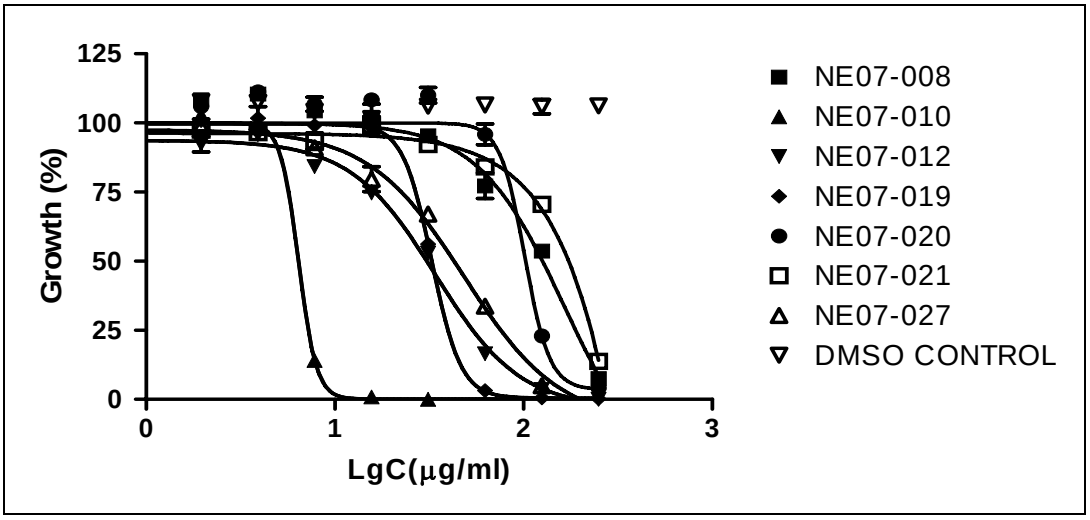


Figure 3: Dose response curves for the seven plant extracts. Note the steep gradient on the dose response curves for 07-010 and 07-019 (from Grevillea *sp.), and the more shallow curves for 07-012 and 07-027 (from* A. flexuosa*)*

Due to their interesting activity profiles, AMI recommended that the four highlighted plant extracts in Table 2 and Table 3 be subjected to chemical isolation to identify the compounds responsible for the antimalarial activity. Bulk material for three of these plant specimens have been collected and extracted (namely 07-010, 07-012 and 07-019) and chemical isolation work has begun. Bulk material for 07-027 (*A. flexuosa* leaves and twigs) will be collected in early April 2009, as this was the time of year that the specimen used for the generation of the screening sample was collected. Particular attention will need to be paid to make sure that if identical chemistry is responsible for the activity of these extracts, it is identified as early as possible so no time is wasted on repeat isolation and structural elucidation of previously purified chemistry.

The results generated from this pilot study were very encouraging. They proved the concept that HPPD could collaborate with AMI and screen natural product extracts for their antimalarial activity. Following this initial pilot screen, AMI made a series of recommendations regarding the future screening of natural products extracts. These include:

- Conduct bioassay guided fractionation of the four active extracts identified in this pilot screen.
- Screen the remaining extract library (124 extracts in total) for *in vitro* antimalarial activity against the D6 cell line of *P. falciparum* using a limited range of concentrations (eg. 500 μg/mL, 50 μg/mL and 5 μg/mL).*

* At the time of composing the technical report this work was still outstanding. Subsequent work in Q1 of the 2008/09 financial year has allowed for this screening work to be completed, and a "hit list" of active extracts to have been compiled.

- Rank these extracts based on their *in vitro* antimalarial activity (for example top 10%, top 20% etc.). This would prioritise the natural product extracts according to their activity profile for further screening.
- Repeat the drug susceptibility assay for extracts selected from the preliminary screening (10-20% of the initial extract pool with highest inhibition at low concentrations) using a 2-fold concentration range in order to establish IC_{50} and IC_{90} values. This data would then be used to select a final prioritised hit list of natural product extracts for future investigation.

The remaining unscreened 124 marine and plant extracts have been prepared and sent for biological testing. The bioassay guided chemical isolation work on the four prioritised specimens will be conducted in financial year 2008/09, under a program of work incorporated into HPPD's DSG task.

2.4 Chemical isolation work

In 2007/08 a Summer Vacation Student (SVS) was employed to aid in the generation of the extract library, to acquire some exposure to the process of natural products chemistry, and to gain experience in the use and operation of spectroscopic equipment including LC-MS and NMR. Initially the SVS processed a batch of 27 plant extracts and conducted the solubility studies. Once these objectives were completed, it was decided that initial isolation work would commence on bulk material of the extracts that were sent to AMI (with the exception of 07-020, as no inflorescence was available). To this end, bulk collections were made (approximately 100 g of each plant part). They were dried in the freeze drier, extracted three times with approximately 500 mL of MeOH, and concentrated *in vacuo* to form a dry gum. Each extract was then submitted to the initial fractionation procedure outlined in Figure 4.

From this analysis, it was clear that the two extracts containing what appeared to be the most interesting chemistry spectroscopically were 07-010 and 07-012. Considering time constraints, it was decided that the remaining extracts would be put on hold and chemical isolation would be concentrated on these two extracts. The aim was to purify and elucidate the structures of as many compounds as possible in the remaining time frame available (approximately 8 weeks). The work conducted on these extracts is discussed below.

2.4.1 Chemical isolation of 07-010

Analysis of the ^{1}H NMR data of the fractions generated from the Solid Phase Extraction (SPE) of 07-010 identified two fractions (Fractions 3 and 4) which gave ^{1}H NMR spectra that were chemically interesting. Each of these fractions was further fractionated using reversed phase semi-preparative C18 HPLC, the chromatograms of which are shown in Figure 5. It is pertinent to point out at this stage the complexity of these semi-purified fractions, with each fraction containing many individual compounds. The aim of the semi-preparative C18 HPLC fractionation step is to isolate pure compounds for structure elucidation using NMR spectroscopy. To this end, fraction three was further fractionated into ten fractions, while fraction four fractionated into six fractions. Subsequent ^{1}H NMR analysis of these HPLC fractions identified two fractions that were pure chemical entities. NMR based structure elucidation of these two pure compounds identified one as the known flavonol quercetin-7-*O*-

[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**),[18] while the second compound was identified as a novel coumaroyl glucose ester (**11**). Arguments pertaining to the structure elucidation of each of these compounds are discussed below.

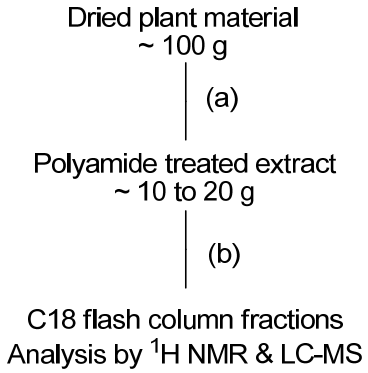


Figure 4: Flow diagram of the general extraction/fractionation procedure applied to the bulk material. (a) Plant material was dried in a freeze drier and extracted with 3 x 500 mL of MeOH. The extract was concentrated and treated with polyamide to remove polyphenolic compounds. (b) Polyamide treated material was then subjected to a C18 Solid Phase Extraction, generating seven fractions. Each of these fractions was further analysed by ^{1}H NMR and LC-MS.

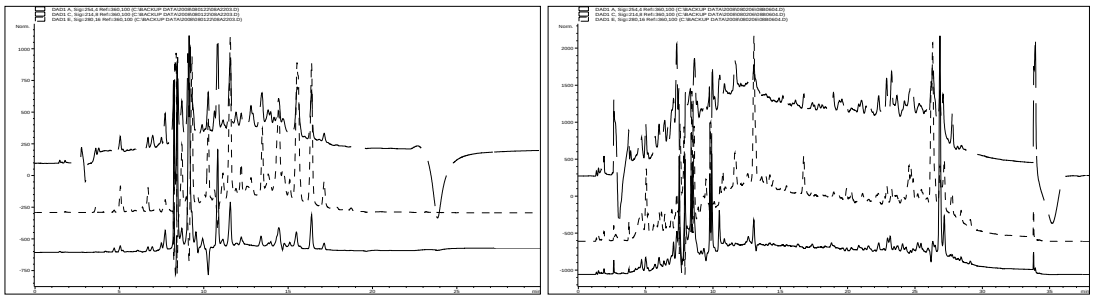
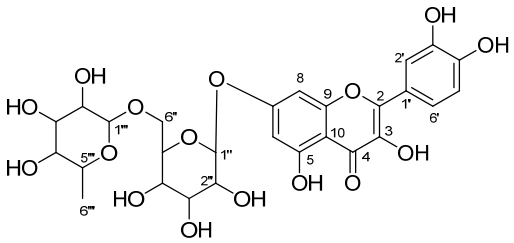


Figure 5: C18 semi-preparative HPLC chromatograms (at 210, 254 and 280 nm) of the two SPE fractions selected for further chemical isolation. Left chromatogram: SPE fraction 3. This fraction was further fractionated into an additional ten fractions, one of which was 10. Right chromatogram: SPE fraction 4. This fraction was further fractionated into an additional six fractions, one of which was 11. Note the complexity still prevalent in the chromatograms, even after SPE.

2.4.1.1 Structure elucidation of quercetin-7-O-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (10)



quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**)

Quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**) was isolated as a bright yellow solid that readily crystallised out of solution. A low mass accuracy mass spectrum suggested that the molecular weight was 610.2 Da ($[M+Na]^+$: 633.2; $[M-H]^-$: 609.1). NMR experiments critical to the elucidation of the structure of **10** were performed, with generated spectra shown in Appendix B. An initial analysis of this NMR data (Table 4) suggested the presence of a compound containing both aromatic and sugar moieties. The observation of carbon resonance in the ^{13}C NMR at 177.4 ppm (C-4) was indicative of a carbonyl carbon. The observation of correlations in the COSY spectrum (Appendix B and Table 4) from H-2' (δ 7.52, d, J = 2.1 Hz) to H-6' (δ 7.54, dd, J = 8.3, 2.1 Hz), and from H-6' to H-5' (δ 6.81, d, J = 8.3 Hz), and gHMBC correlations (Appendix B and Table 4) from H-2' into C-4' (148.5 ppm) and C-6' (121.6 ppm), from H-5' to C-1' (121.2 ppm) and C-3' (144.7 ppm), and from H-6' to C-4' and C-2' (116.2 ppm) allowed for the identification of a tri-substituted aromatic moiety with hydroxyl groups pendant at C-3' and C-4'. Further gHMBC correlations from H-2' and H-6' into a carbon C-2 (156.6 ppm) indicated that the tri-substituted aromatic moiety was substituted at C-2. Furthermore, it was apparent from this data of the presence of an α,β unsaturated ketone system, with the resonance of C-3 (133.3 ppm) consistent with hydroxyl substitution. These NMR data allowed for part structure A to be elucidated as shown in Figure 6.

Part structure B was elucidated through the observation of the following important correlations. The observation of weak COSY correlation between H-6 (δ 6.15) and H-8 (δ 6.34), as well as the magnitude of the coupling constant (1.9 Hz) was evidence that these two protons were *meta* coupled aromatic protons. This was further supported through the observation of gHMBC correlations from H-6 to C-8 (93.6 ppm), and from H-8 to C-6 (98.7 ppm). In the absence of any other aromatic protons, it was apparent that this part structure was a tetra-substituted aromatic ring. Furthermore, the ^{13}C chemical shifts of C-6 and C-8 were evidence for each of them being *ortho* to two carbons substituted with oxygens. The magnitude of the ^{13}C chemical shift for C-5 (161.2 ppm), C-7 (164.2 ppm) and C-9 (156.6 ppm) was evidence for further oxygen substitution at these carbons. Hence part structure B was established to be as shown in Figure 6.

The presence of two anomeric carbons C-1'' and C-1''' indicated that **10** contained two sugar moieties. Furthermore, the observation of a methyl resonance in the NMR data at δ 0.98, (d, 6.8Hz) and ^{13}C 17.8 ppm (C-6'''), with COSY and gHMBC correlations into C-5''', and a gHMBC correlation into C-4''' suggested that one of the sugar moieties was a rhamanose.

Table 4: NMR data (500 MHz, d_6-DMSO) for 10

No	^{13}C (δ, m)	^{1}H (δ, m, *J* (Hz))	COSY	gHMBC
1				
2	156.5 (s)[a]			
3	133.3 (s)			
4	177.4 (s)			
5	161.2 (s)			
6	98.7 (d)	6.15 (d, 1.9)	H-8	C-5, C-7, C-8, C-10
7	164.2 (s)			
8	93.6 (d)	6.34 (d, 1.9)	H-6	C-6, C-7, C-9, C-10
9	156.6 (s)[a]			
10	103.9 (s)			
1'	121.2 (s)			
2'	116.2 (d)	7.52 (d, 2.1)	H-6'	C-2, C-1', C-3', C-4', C-6'
3'	144.7 (s)			
4'	148.5 (s)			
5'	115.2 (d)	6.81 (d, 8.3)	H-6'	C-1', C-3'
6'	121.6 (d)	7.54 (dd, 8.3, 2.1)	H-2', H-5'	C-2, C-2', C-4'
1''	101.2 (d)	5.31 (d, 7.4)	H-2''	
2''	74.1 (d)	3.21 (m)	H-1''	C-1'', C-4''
3''	70.3 (d)	3.38 (m)		
4''	76.4 (d)	3.21 (m)		C-2''
5''	70.0 (d)	3.05 (m)	H_a-6''	C-3''
6''	67.0 (t)	3.69 (d, 10.1) 3.27 (m)	H_b -6'' H-5, H_a-6''	C-1'''
1'''	100.8 (d)	4.37 (br s)		C-3''', C-5'''
2'''	75.9 (d)	3.23 (m)		
3'''	70.5 (d)	3.28 (m)		
4'''	71.9 (d)	3.06 (dd, 9.4)	H-3''', H-5'''	C-5'''
5'''	68.3 (d)	3.27 (dq, 9.4, 6.7)	H-4''', H-6'''	
6'''	17.8 (q)	0.98 (d, 6.7)	H-5'''	C-4''', C-5'''
5-OH		12.57 (br s)		

[a] Chemical shifts may be interchanged

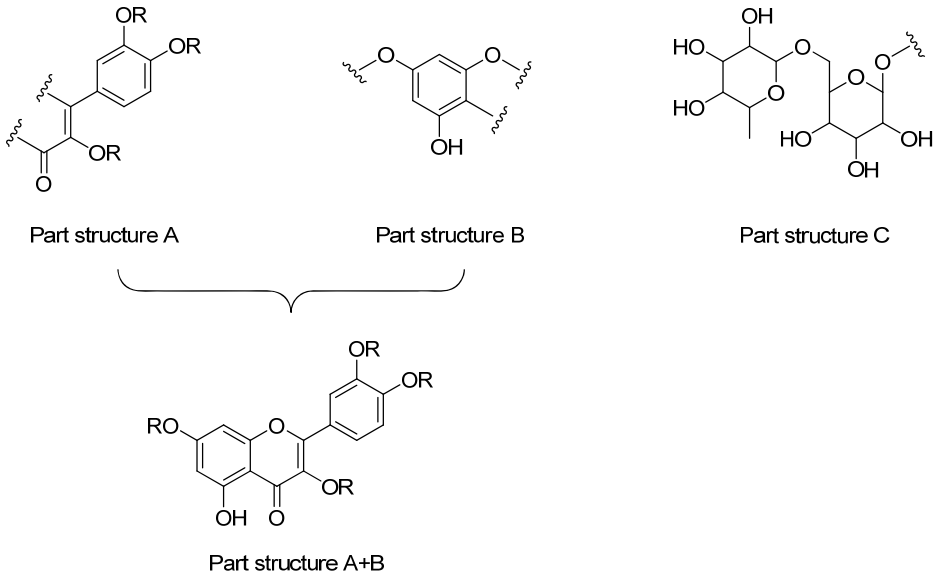
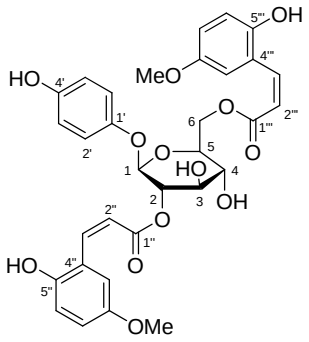


Figure 6: Elucidated part structures A to C for 10

From the remaining NMR data it was apparent that the other sugar was glucose. The observation of a gHMBC correlation from Hb-6'' to C-1'' identified that the two sugar moieties, and hence part structure C, were part of a disaccharide, as shown in Figure 6.

The three part structures were linked through the following observations and key correlations. The observation of a ^{1}H NMR resonance at δ 12.57 (5-OH, br s) is indicative of a hydroxyl proton hydrogen bonding to a carbonyl oxygen. Additionally, the ^{13}C chemical shift of C-2 (156.5 ppm) and C-3 is indicative of the α and β positions of an unsaturated ketone spin system as part of a flavonol ring. This allows part structure A and B joined forming the flavone ring system shown in Figure 6. The remaining part structure C (the disaccharide) could attach at any of the four remaining positions. With no clear spectroscopic evidence to support any of the positions, a SciFinder sub-structure search was conducted to aid in the identification of the structure of **10**. This search yielded one compound[18] with a structure that seemed to explained the observed NMR data for **10**. Comparison of the collected NMR data for **10** with that reported in the literature readily identified the structure of **10** as the known flavonol quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside].

2.4.1.2 Structure elucidation of the coumaroyl β-glucose ester (11)



coumaroyl glucose ester (**11**)

The coumaroyl β-glucose ester (**11**) was isolated as a brown oil, with low mass accuracy mass spectral data ($[M+Na]^+$ = 647.2; $[M-H]^-$ = 623.2) indicating a molecular weight of 624.2 Da. A comparison of the NMR data for quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**) with **11** (Table 5 and Appendix C for gHMQC, COSY and gHMBC spectra) showed little resemblance, suggestive of **11** being a different structure class to **10**. Additionally, it was apparent from the NMR data that there was a high degree of symmetry for **11**, with the presence of a complicated aromatic region in the ^{1}H NMR spectrum that seemed to have duplicated signals, as highlighted by the red box in Figure 7. Evident in the ^{1}H NMR spectrum for **11** was resonances (blue box Figure 7) consistent with a sugar moiety. This was confirmed through the observation of ^{1}H (δ 4.95) and ^{13}C (102.4 ppm) resonances that were consistent with an anomeric carbon (C-1, see Table 5). The identification of a contiguous spin system from C-1 to C-6 through COSY and gHMBC correlations (Table 5), together with the magnitude of the coupling constants for H-1 to H-6 (large *J* values indicative of axial-axial couplings), established the sugar as β-glucose (Figure 8, part structure A). Furthermore the observed NMR chemical shifts of C-2 (^{1}H: δ 5.09; ^{13}C: 75.1 ppm) for **11** was consistent with an ester substitution at C-2.

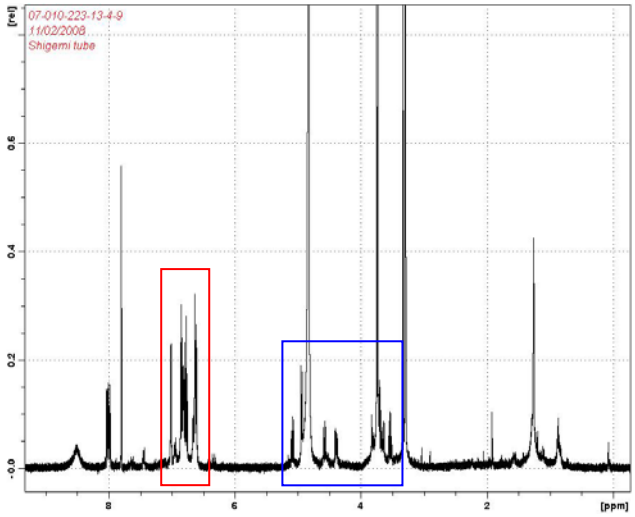


Figure 7: ^{1}H NMR spectrum for 11. Notice the complexity of the aromatic region of the spectrum highlighted by the red box.

The observation of COSY correlations from H-2'/6' (δ 6.86) to H-3'/5' (δ 6.62), with additional gHMBC correlations from H-2' into C-4' (153.7 ppm) and C-6' (119.9 ppm), from H-3' into C-1' (151.9 ppm) and C-5' (116.9 ppm), from H-5' into C-1' and C-3' (116.9 ppm) and from H-6' into C-2' and C-4' allowed for part structure B (Figure 8) to be identified as a para-disubstituted aromatic group. The magnitude of the ^{13}C chemical shifts of C-1' and C-6' also indicated oxygen substitution at these positions.

Observed COSY correlations from H-2''/2''' (δ 6.61) to H-3''/3''' (δ 7.98, δ 8.03) with additional gHMBC correlations from H-3''/3''' to C-1''/1''' (168.3 ppm, 169.2 ppm) established two α,β-unstaturated carbonyl spin systems for part structures C and D. Further gHMBC correlations from H-2''/2''' to the aromatic carbons C-4''/4''' (123 ppm), and from C-3''/3''' to two further aromatic carbons C-5''/5''' (152.8 ppm) and C-9''/9''' (113.5 ppm) implied that part structures C and D were in fact coumaroyl esters (Figure 8). The magnitude of the coupling constant between H-2''/2''' and H-3''/3''' (J = 10.7 Hz) established a Z geometry for the C-2''/2'''-C-3''/3''' double bond. Further COSY correlations from H-6''/6''' (δ 6.78) to H-7''/7''' (δ 6.82) and from H-7''/7''' to H-9''/9''' (δ 7.05), in addition to the magnitude of the coupling constants for H-6''/6''', H-7''/7''' and H-9''/9''' established that the aromatic groups of the coumaroyl esters were 1,2,4-trisubstitued. This substitution pattern was further supported through the observed gHMBC correlations outlined in Table 5. The methoxy groups were positioned at the C-8''/8''' positions through the observation of gHMBC correlations from 8-OMe''/8-OMe''' into C-8''/8'''. Hence part structures C and D were established to be the substituted coumaroyl esters shown in Figure 8.

The observation of gHMBC correlations from H-1 into C-1' positioned the para-disubstituted aromatic ring at C-1, while gHMBC correlations from H-2 into C-1'' and H_2-6 in C-1''' positioned the two coumaroyl esters at C-2 and C-6. Taking into account the observed molecular weight of 624.2 Da, these observations accounted for 621 Da. With three remaining points of attachment (at oxygen substitutions at C-4', C-5'' and C-5''') and 3 Da still remaining to be explained, it was immediately apparent that these were in fact hydroxyl groups.

Therefore the gross structure of **11** is elucidated as shown. Significantly, sub-structure searches for the elucidated structure of **11** identified it to be a novel compound.

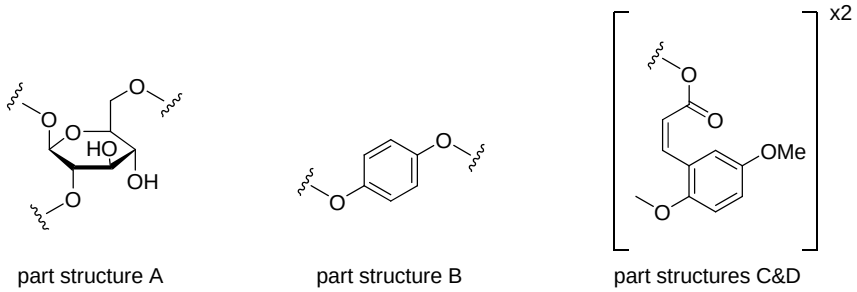


Figure 8: Elucidated part structures for 11

Table 5: NMR data (500 MHz, d_4-MeOH) for 11

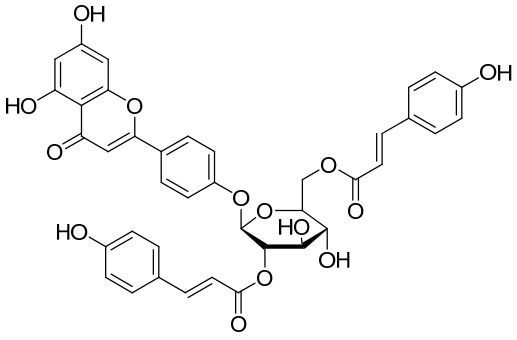
No	^{13}C (δ, m)[a]	^{1}H (δ, m, *J* (Hz))	COSY	gHMBC
1	102.4 (d)	4.95 (d, 8.0)	H-2	C-1', C-2
2	75.1 (d)	5.09 (t, 8.0)	H-1, H-3	C-1, C-3, C-1''
3	76.1 (d)	3.70 (m)	H-2, H-4	C-2, C-5
4	71.9 (d)	3.54 (t, 9.0)	H-3, H-5	C-5, C-6
5	75.6 (d)	3.74 (m)	H-4, H_2-6	
6	64.7 (t)	4.58 (d, 11.2) 4.39 (dd, 11.2, 6.6)	H-5, H_b-6 H-5, H_a-6	C-1''' C-5, C-1'''
1'	151.9 (s)			
2'	119.9 (d)	6.86 (d, 8.8)	H-3'	C-4', C-6'
3'	116.9 (d)	6.62 (d, 8.8)	H-2'	C-1', C-5'
4'	153.7 (s)			
5'	116.9 (d)	6.62 (d, 8.8)	H-6'	C-1', C-3'
6'	119.9 (d)	6.86 (d, 8.8)	H-5'	C-2', C-4'
1''	168.3 (s)			
2''	117.0 (d)	6.61 (d, 10.7)	C-3''	C-4''
3''	142.7 (d)	7.98 (d, 10.7)	C-2''	C-1'', C-2'', C-5'', C-9''
4''	123.0 (s)			
5''	152.8 (s)			
6''	118.0 (d)	6.78 (d, 9.1)	C-7''	C-4'', C-8''
7''	119.8 (d)	6.82 (dd, 9.1, 2.1)	C-6'', C-9''	C-5'', C-9''
8''	154.4 (s)			
9''	113.5 (d)	7.02 (d, 2.1)	C-7''	C-3'', C-5'', C-7''
8''-OMe	49.5 (q)	3.76[b] (s)		C-8''
1'''	169.2 (s)			
2'''	117.0 (d)	6.61 (d, 10.7)	C-3'''	C-4'''
3'''	142.3 (d)	8.03 (d, 10.7)	C-2'''	C-1''', C-2''', C-5''', C-9'''
4'''	123.0 (s)			
5'''	152.8 (s)			
6'''	118.0 (d)	6.78 (d, 9.1)	C-7'''	C-4''', C-8'''
7'''	119.8 (d)	6.82 (dd, 9.1, 2.1)	C-6''', C-9'''	C-5''', C-9'''
8'''	154.4 (s)			
9'''	113.5 (d)	7.02 (d, 2.1)	C-7'''	C-3''', C-5''', C-7'''
8'''-OMe	49.5 (q)	3.74[b] (s)		C-8''

[a] *^{13}C NMR chemical shifts obtained from gHMQC and gHMBC experiments.*
[b] *Chemical shifts may be interchanged*

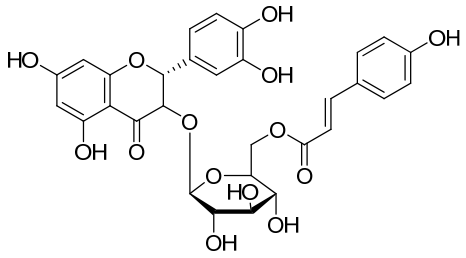
It should be noted at this time that the elucidated gross structure for **11** is currently tentative. To complete the structure elucidation an accurate mass measurement needs to be made using

the FTMS. At the time of writing this report the new instrument has yet to be installed, precluding the inclusion of the data in this report. It should also be highlighted here that as this is a previously unreported compound as soon as all data has been collected and interpreted it will be written up for publication in the peer reviewed literature.

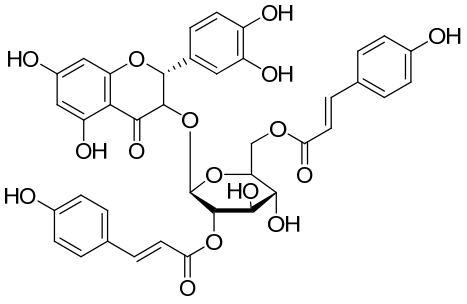
There are several literature reports of compounds related to **11** containing a flavonoid substituent at C-1 with coumaroyl esters substituted at either or both of C-2 and C-6,[19-27] as represented by apigenin-4'-*O*-(2'',6''-di-*O*-*p*-coumaroyl)-β-D-glucoside (**12**) from *Lycopodium clavatum*,[25] and catechin-3-*O*-β-D-gluco(2-cinnamoyl)pyranoside (**13**) and catechin-3-*O*-β-D-gluco(2,6-biscinnamoyl)pyranoside (**14**) from *Inga umbellifera*.[26] This is the first reported structure to have phenolic substitution at C-1, as well as the first report of this class of compound being isolated from a *Grevillea* sp.



(**12**)



(**13**)



(**14**)

2.4.1.3 Further work for 07-010

While two compounds have been isolated from the crude extract of 07-010, as it currently stands, it is unclear if they account for the observed activity of the crude extract. Considering the considerable activity observed for 07-010, bioassay guided fractionation of the crude extract has commenced to identify the chemical constituent(s) responsible for the activity. Once completed, a chemical and biological assessment of all isolated compounds from 07-010 will be made to establish their suitability for further investigations.

In addition to the collection of FTMS data for **11**, the absolute stereochemistry of the β-glucose also needs to be established through hydrolysis to yield the β-glucose. A comparison of the

optical rotation ($[\alpha]_D$) of the hydrosylate with an authentic standard of D-glucose will establish the stereochemistry of the β-glucose from **11** as either D- or L-, and hence determine the absolute stereochemistry of **11**.

2.4.2 Chemical isolation of 07-012

Partial isolation of 07-012 was undertaken during the tenure of the SVS student. Using the strategy for isolation shown in Figure 4, bulk extract of 07-012 was extracted and subjected to a C18 flash column. Following fractionation, ^{1}H NMR spectra were collected for each fraction. This analysis identified several fractions (see Appendix D) as having interesting chemistry. However, due to time constraints no further work could be completed on these extracts by the SVS student.

The extract was also identified as having reasonable antimalarial activity so further bioassay guided isolation work will be conducted to identify and solve the structure of the active chemical compound(s).

3. Conclusions

The overall aim of this mLRR project was to assess the feasibility of conducting natural products based drug lead discovery work for Defence relevant disease states. Initially focussed on antibacterial discovery work, the focus of the project changed due to unforeseen circumstances to developing a library of natural product extracts to be screened at a later date. Through contacts established at AMI an opportunity presented itself to screen a small selection of plant samples (seven) *in vitro* against the D6 line of *P. falciparum*. Of these it was found that four extracts had selective activity against the malaria parasites, leaving the red blood cells intact and viable. In addition, chemical investigations into the extract of a *Grevillea* sp allowed for the isolation of two compounds, one of which appears to be a new compound.

In a short period of time, a library of extracts from 131 specimens made up of various plants and marine sponges was established. These specimens were processed to produce extracts for screening, and were chemically fingerprinted using ^{1}H NMR and LC-MS. This was to aid in dereplication of active extracts as required. Also demonstrated was the capacity of isolating from a bulk extract pure compounds and elucidating their chemical constitution in reasonable time frames.

Considering the success of this mLRR, it is felt that there may be longer term opportunities in lead discovery space that HPPD may be able to leverage in the Defence health area. This could also be expanded into the civilian first responders and the broader civilian community. These potential therapeutic areas of interest include:

- Antibacterial agents. In particular against potential biological warfare agents such as the etiological agent of anthrax, *Bacillus anthracis*. Considering the resistance that has been acquired by bacteria to existing classes of drugs and the distinct lack of novel antiinfectives released to the market in recent years for many reasons (including a withdrawal of resources by pharmaceutical companies due to the perceived difficulty

in finding novel patent space, and hence cost effectiveness), the need to discover and develop new antibiotics with novel modes of action is critical.[28]

- Multi-drug resistance (MDR). This has implications in most relevant disease states. Treatments that are able to switch off MDR pathways (such as efflux pumps) convey additional longevity to existing therapeutics. Existing treatments to disease states that have high degrees of resistance such as malaria and bacterial infections could benefit from research targeting this area.
- Melioidosis is a significant potential health issue for military personnel deployed to tropical regions during the wet season. Caused by the bacteria *Burkholderia pseudomallei*, it can have long latency period, and is a potential biological warfare agent.
- Other vector borne diseases. These include such diseases as Dengue fever, Ross River fever, Leishmania, Chugas disease, African sleeping sickness and Trypansomes, and can have a potentially significant impact on deployed personnel.
- Filoviruses which cause severe hemorrhagic fever. There are two members of this class of virus, namely the Ebola and Marburg viruses.
- Other ADF related health states as needs and priorities arise (such as analgesics, cholera and dysentery).

The ADF has a unique set of health and disease states that its personnel may encounter. They are often deployed to countries/regions in the world with endemic diseases that are not present in Australia. These include vector born (such as malaria) and water born disease (such as cholera and dysentery). Additional to this, there are bioterrorism threats of anthrax (caused by *B. anthracis*) and melioidosis (caused by *B. pseudomallei*) to which both the ADF and the civilian population may be exposed. Several of these disease states are not well catered for by existing industry/academic research programs. This mLRR project has shown that HPPD chemistry capability is well placed to establish a niche research area and deliver quality research to clients in DSG/DHS.

4. Experimental

4.1 General Experimental

The solvents used (MeOH, ACN, H_2O and formic acid) were HPLC grade and obtained from Merck. Solid phase (C18 and polyamide) for column chromatography was obtained from Phenomenex. High Pressure Liquid Chromatography (HPLC) was performed on an Agilent 1100 series LC system comprising of an in-line degasser, binary pump, auto-injector, column heater and diode array detector and fraction collector. Spectroscopic data was collected using the Agilent ChemStation LC for 3D software (Rev.A.09.03).

NMR data was collected on a Bruker Avance 500 MHz spectrometer using deuterated NMR solvents supplied by Cambridge Isotopes. Spectra were referenced to residual ^{1}H and ^{13}C in the deuterated solvents.

LC-MS data was collected on an Agilent LC/MSD Trap XCT mass spectrometer connected to an Agilent 1100 series LC system comprising of an in-line degasser, binary pump, auto-injector, column heater and diode array detector, equipped with Agilent ChemStation LC for 3D software (Rev.A.09.03). Samples were eluted through a Phenomenex Gemini 5 μm 50 mm x 2.0 mm C18 HPLC column, using gradient elutions from 5% MeOH in H_2O (+ 0.05% formic acid) to 100% MeOH + 0.05% formic acid over 35 min.

4.2 Isolation of 10 and 11 from 07-010 (*Grevillea* sp.)

Bulk plant material (100 g) was freeze dried, ground and extracted three times with 500 mL of MeOH. The combined MeOH extracts were concentrated *in vacuo* and resuspended in approximately 20 mL of MeOH. The solution was treated with polyamide to remove any polyphenolic compounds, and eluted off the column with approximately 500 mL of MeOH. The MeOH eluant was concentrated again *in vacuo* and then subjected to reversed phase C18 flash vacuum column with gradient elution from 0%, 20%, 50%, 70%, 90% and 100% MeOH in H_2O. Subsequent ^{1}H NMR analysis identified interesting resonances in the 50% and 70% MeOH in H_2O fraction. This material was then further fractionated using C18 semi-preparative HPLC (gradient elution from 20% ACN in H_2O (+ 0.05% formic acid) to 100% ACN (+ 0.5% formic acid) over 30 min, through a C18 Phenomenex Luna 150 mm x 10 mm HPLC column). Further analysis by ^{1}H NMR identified that fraction one contained the known compound quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**, > 400 mg), while fraction nine contained the novel coumaroyl glucose ester (**11**, 1.1 mg).

4.3 Antimalarial biological screening protocols

Parasite cultures were maintained in complete LPLF RPMI media supplemented with 10% human plasma at 4% hematocrit.[15] Cultures were treated with D-sorbitol[16] every 48 h to produce highly synchronous experimental cultures. Two methods were used to evaluate the *in vitro* antimalarial activity of the plant extracts: inhibition of ^{3}H-hypoxanthine uptake using 48 h and 96 h exposure[17] and microscopic analysis after 48 h exposure.

The plant extracts were dissolved in DMSO to the concentration of 0.1 mg/μl (or 100 mg/ml). For the initial screen, three 10-fold dilutions of the stock were made using hypoxanthine-free complete media containing 10% human plasma to achieve final concentrations in the well of 500, 50 and 5 μg/ml. Fifty μl of the respective extract dilutions were added to the wells of 96-well microtitre plates and mixed with 50 μl of inoculum containing ring stages (6-12 hours post invasion) of D6 parasites at 1% parasitemia and 4 % hematocrit (final 2%) prepared in hypoxanthine-free complete media. In addition, the same dilutions of the solvent (DMSO) were made and tested alongside the plant extracts to rule out the inhibitory effect from the solvent. Duplicate plates were made for the microscopic assessment of parasite growth versus extract concentration.

The plates were incubated at 37 °C for ~24 h until parasites reached the trophozoite stage, then 20 μl of ^{3}H-hypoxanthine diluted 100-fold in RPMI plain media was added to each well and the plates were incubated for a further 24 or 72 h. The hypoxanthine uptake assay plates were frozen and processed as described elsewhere.[17] The *in vitro* antimalarial activity of the plant extracts is expressed as inhibition of parasite growth measured as the ratio of

^{3}H-hypoxanthine uptake in the wells containing plant extracts to that in the control wells (no extract).

Additionally, for estimating the concentration of the plant extracts which inhibits the parasite growth by 50%, D6 parasites were exposed to ten 2-fold dilutions of the extracts with concentrations starting from 250 µg/ml to 0.24 µg/ml using the same *in vitro* conditions described above. IC_{50} values were defined as the extract concentrations producing 50% inhibition of uptake of ^{3}H-hypoxanthine by D6 parasites compared to extract-free samples (controls). In parallel to the hypoxanthine uptake study of the extracts, chloroquine was used as a reference drug.

For microscopic analysis, thick blood smears were made and stained with Giemsa. In addition, thin smears from the wells with highest concentration (500 µg/ml) of the plant extracts were made to rule out the toxic effect of the extracts on red blood cell membrane integrity.

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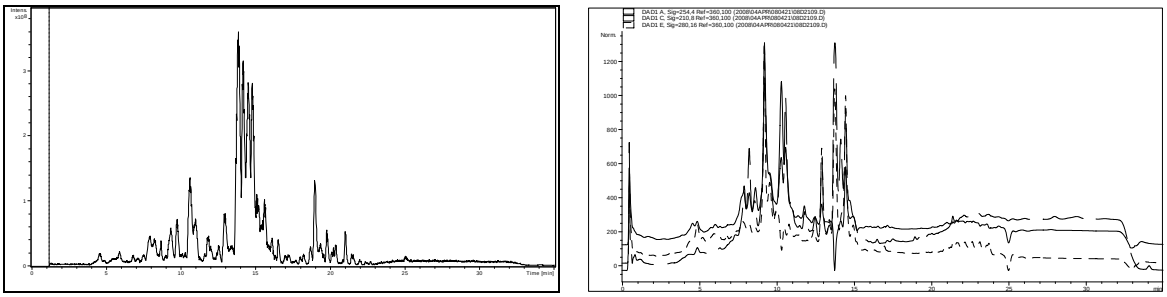
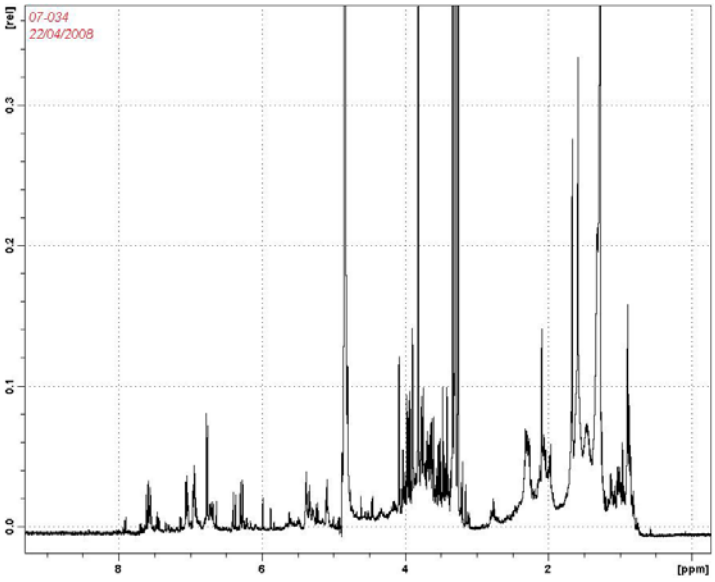
Appendix A: Representative LC-MS and ^{1}H NMR spectra data for chemical fingerprinting

Shown below are representative chemical fingerprint data collected for a plant (07-034) and a marine (08-M017) specimen. These data identify the complexity inherent in natural product extracts, and the challenge a chemist is faced with when isolating small amount of material from a complex matrix of compounds.

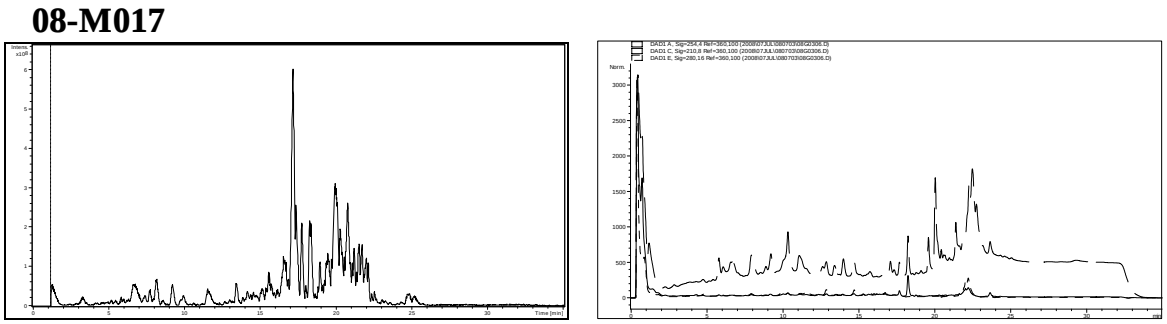
07-034



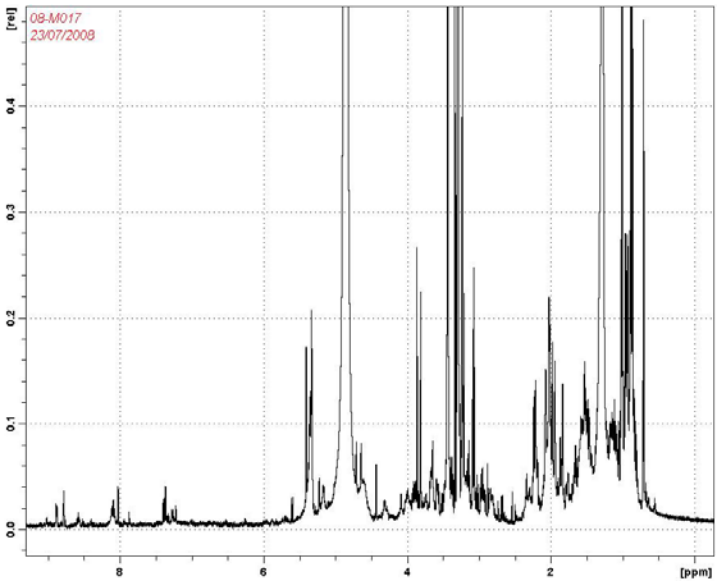
The LC-MS (left hand spectrum) and HPLC-UV chromatograms (right hand spectrum at 210, 254 and 280 nm) of the plant extract 07-034. These data are collected at the same time, with common retention times in each analysis identifying the same compound. As can be seen many compounds are identified at the one time.



The ^{1}H NMR spectrum of the crude extract of 07-034. Although a less sensitive technique than LC-MS, it is immediately apparent from this analysis the complexity of chemistry present in the plant extract. NMR spectroscopy is the most powerful analytical technique for elucidating the structure of unknown compounds.



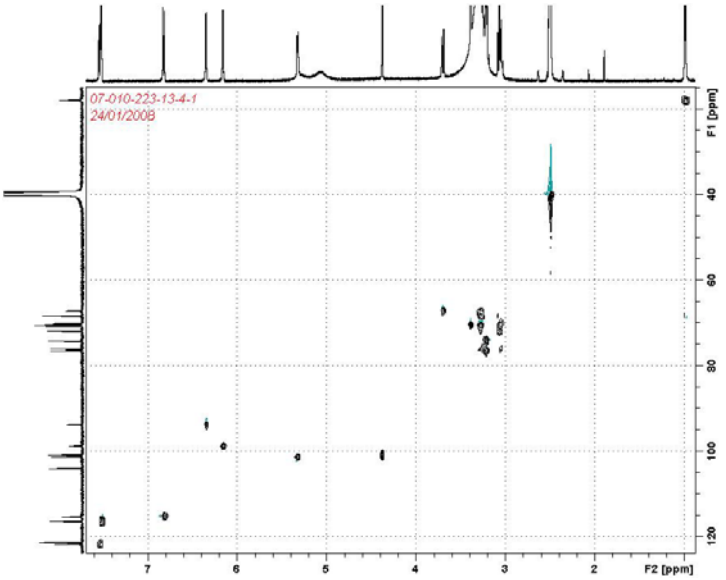
The LC-MS (left hand spectrum) and HPLC-UV chromatograms (right hand spectrum at 210, 254 and 280 nm) of the marine extract 08-M017.



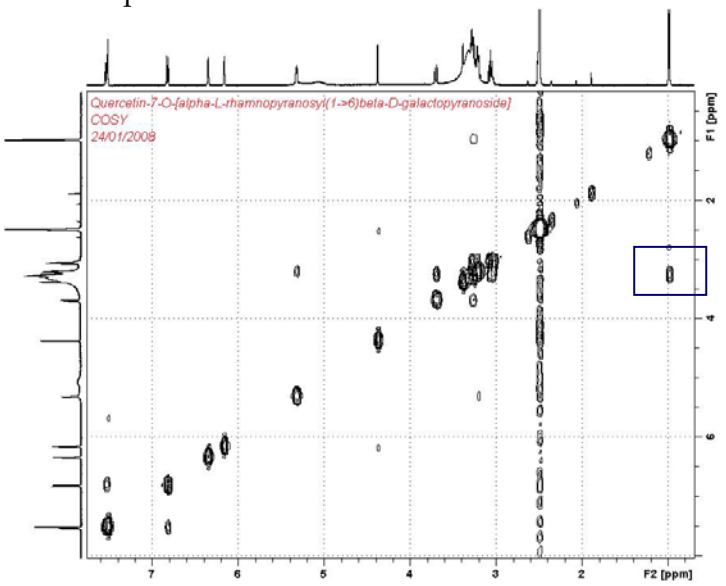
The ^{1}H NMR spectrum of the crude extract of 08-M017.

Generally, marine extracts tend to be more non-polar than plant extracts, and hence have later eluting compounds of HPLC, and more signals in the 0.8-3.0ppm range in the ^{1}H NMR. This is reflected in the data shown for the two different extract types discussed here.

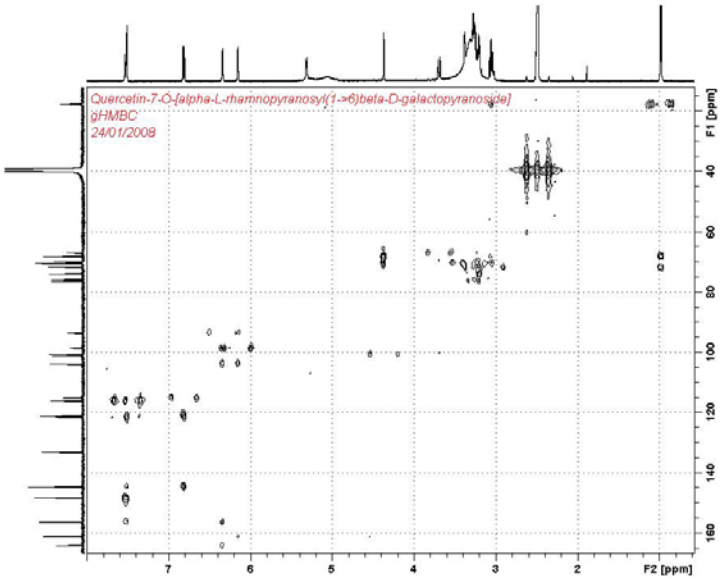
Appendix B: Two dimensional NMR spectra for 10



The spectrum above is the data generated from a gHSQC experiment run on quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**). Each cross peak shown above represents a direct ^{1}H-^{13}C correlation and is essential data when elucidating the structure of complex unknown natural products.

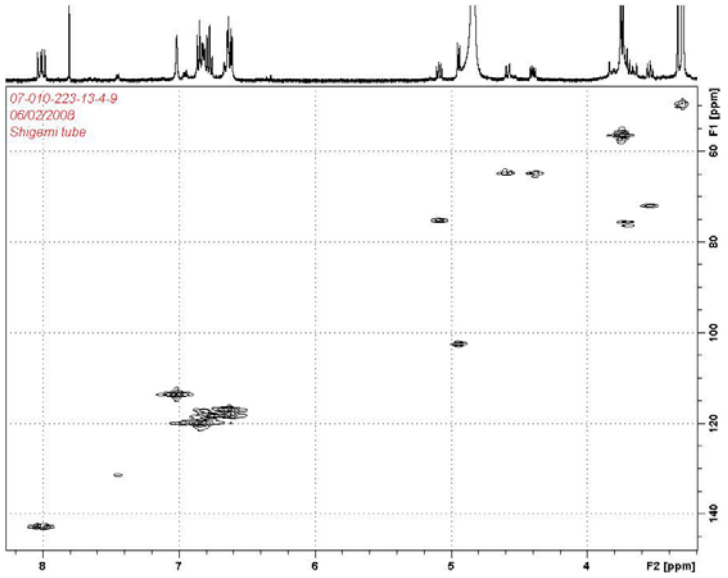


The spectrum above is the data generated from a COSY experiment run on quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**). Each of the cross peaks shown above (an example of which is highlighted in the blue box) allows for a determination of which protons in the molecule are next to each other. This data, coupled with the direct ^{1}H-^{13}C correlations established from the gHSQC experiment, allows for small pieces of the unknown compound to be determined.

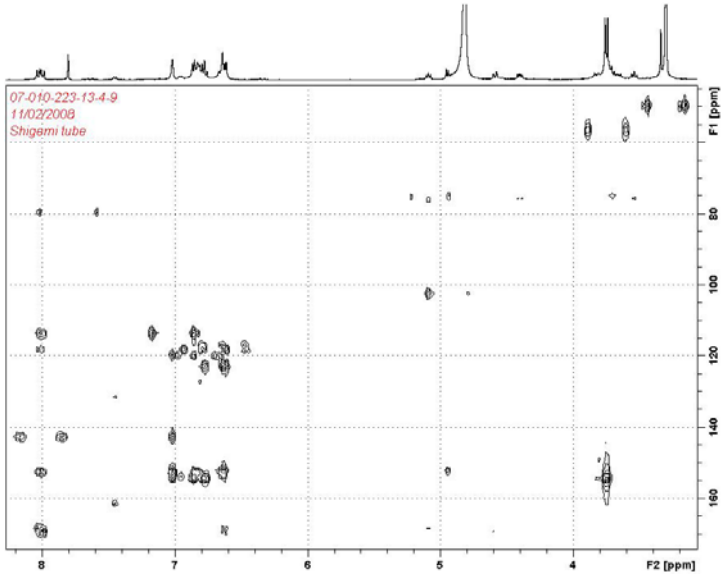


The spectrum above is the data generated from a gHMBC experiment run on quercetin-7-*O*-[α-L-rhamnopyranosyl(1->6)-β-D-galactopyranoside] (**10**). Each cross peak shown above represents either a 2 bond or 3 bond ^{1}H-^{13}C correlation. These data allows for links between all of the smaller pieces of the molecule that have been identified in the gHSQC and COSY spectra to be established.

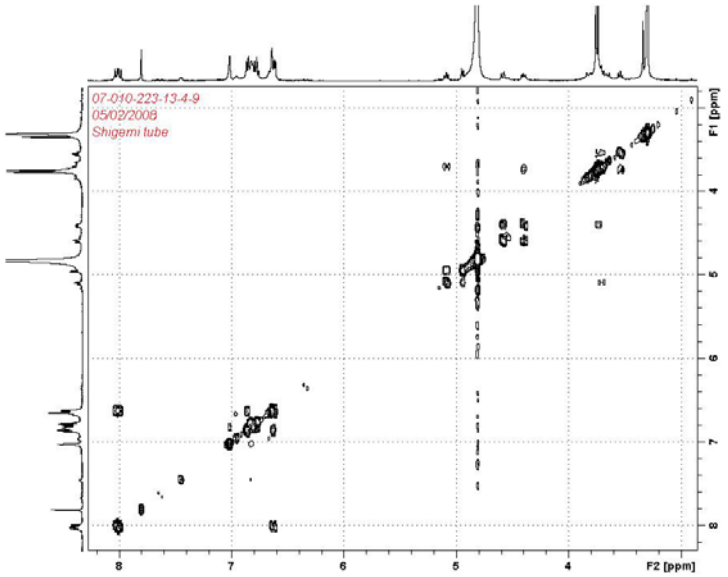
Appendix C: Two dimensional NMR spectra for 11



The gHMQC spectrum (identical to a gHSQC experiment) obtained for the coumaroyl β-glucose ester (**11**). Due to the amounts of compound available for **11** (approximately 1 mg), it was decided to run this experiment as it is more sensitive. Of interest is that the correlations are significantly broader for the gHMQC experiment, as compared to the gHSQC spectrum generated for **10** (as shown in Appendix B above).

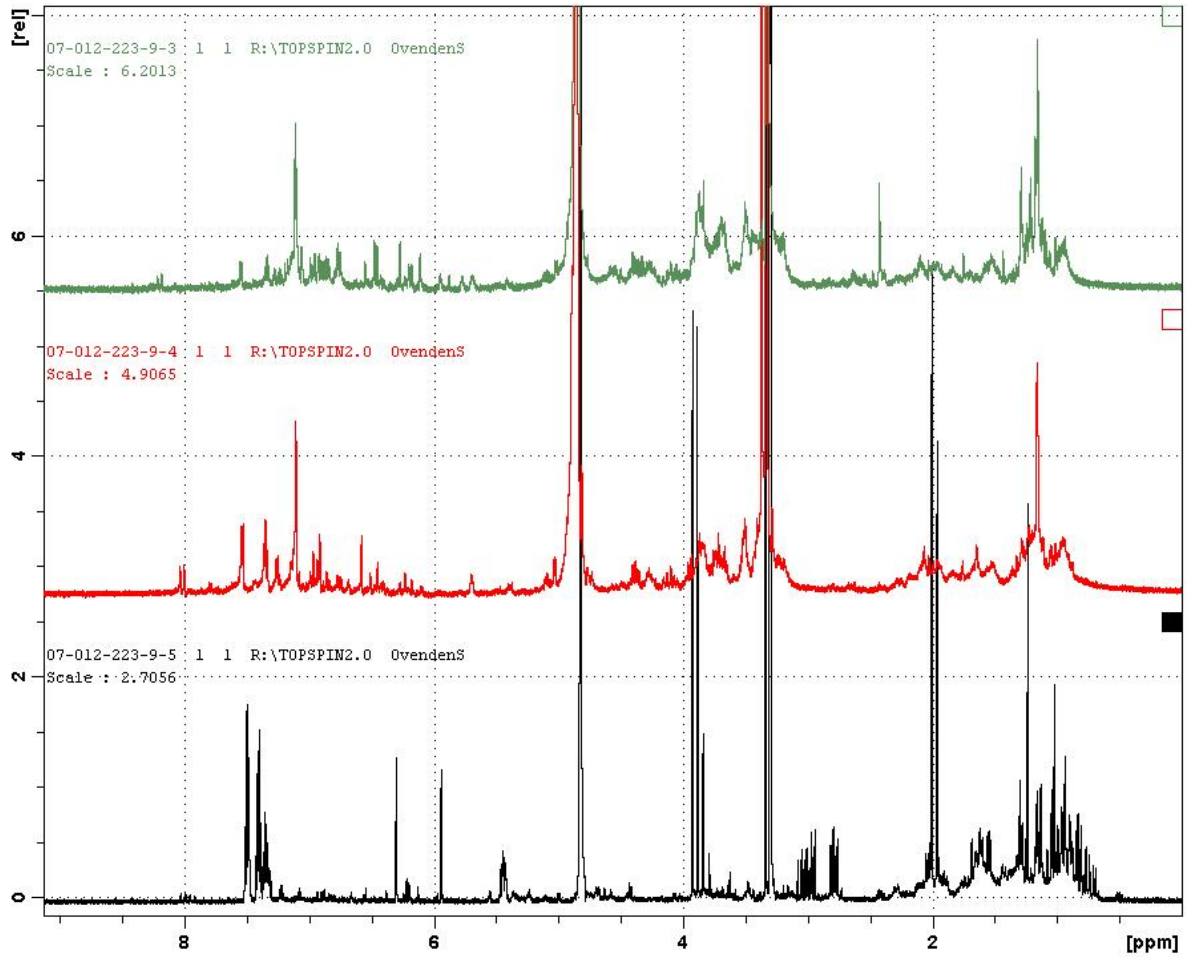


The gHMBC spectrum obtained for the coumaroyl β-glucose ester (**11**).



The COSY spectrum obtained for the coumaroyl β-glucose ester (**11**).

Appendix D: Stack plot of the ^{1}H NMR spectra of selected fractions from 07-012



Stack plot of the three most interesting ^{1}H NMR spectra that were obtained from the C18 flash column of the plant extract 07-012 (*Agonis flexuosa*). Green spectrum: fraction 3; red spectrum: fraction 4; black spectrum: fraction 5.

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19. ABSTRACT

This technical report outlines the research activites conducted during a mini Long Range Research project to establish a natural product extract library for drug disocvery. The library was established with a view to it being screened against Defence health related targets. Discussed in this report are the research results generated from this project including establishing and chemically fingerprinting the extract library, *in vitro* results from screening a small selection of the library for antimalarial activity against parasites of *Plasmodium falciparum*, and the elucidation of the chemical structures of two compounds. The viability of conducting this type of research within the Human Protecton and Performance Division at DSTO is also discussed.