An ethnobotanical, ecological and LC-MS-based chemometric investigation of Phaleria nisidai, a traditional adaptogen containing diterpene esters from Palau, Micronesia

Daniel Kulakowski

Graduate Center, City University of New York

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An ethnobotanical, ecological and LC-MS-based chemometric investigation of *Phaleria nisidai*, a traditional adaptogen containing diterpene esters from Palau, Micronesia

by

Daniel Kulakowski

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2014
This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Date

Chair of Examining Committee
Dr. Edward J. Kennelly, Lehman College

Date

Executive Officer
Dr. Laurel A. Eckhardt

Dr. Dwight Kincaid, Lehman College

Dr. Laurel A. Eckhardt, Hunter College

Dr. Michael J. Balick, The New York Botanical Garden

Dr. Jianda Yuan, Memorial Sloan-Kettering Cancer Ctr.

Supervising Committee

The City University of New York
Abstract

An ethnobotanical, ecological and LC-MS-based chemometric investigation of *Phaleria nisidai*, a traditional adaptogen containing diterpene esters from Palau, Micronesia

by Daniel Kulakowski

Advisor: Dr. Edward J. Kennelly

Palau is a country with a rich heritage of traditional medicine still being practiced. One of the most popular and respected remedies in Palau is a tea made from fresh leaves of *Phaleria nisidai* Kaneh. (Thymelaeaceae). Interviews conducted to determine the use of this plant revealed that it is employed non-specifically to treat a variety of general health concerns. Its use as a prophylactic to keep away sickness, as a ‘system cleaner’, as well as for strength and energy indicate that it is being used as an adaptogen, a medicine taken routinely to help adapt to external pathogenic, mental or physical stress. A series of *in vitro* immunological assays were conducted to determine the effect of crude extracts and guide phytochemical fractionation of this plant to identify immunostimulant compounds.

*Phaleria nisidai* is in the family Thymelaeaceae, one of two plant families known to produce diterpene ester compounds. Multivariate analysis to compare mass spectral data of active and inactive fractions allowed for the identification of several daphnane diterpene esters as tentative active marker compounds. Simplexin, the marker contributing most to differentiation of active and inactive fractions, was active when tested alone for stimulation of cytokine output by peripheral blood mononuclear cells (PBMCs). This demonstrated that a chemometric approach to active compound determination as opposed to traditional time- and resource-intensive bioactivity-guided purification methods to determine active compounds in a plant matrix was successful. This work also confirms a methanol extract of this plant and one of its constituents
has activity as an immunostimulant, verifying its traditional use. This is also the first report of diterpene esters in the genus *Phaleria*.

Simplexin and other diterpene esters have shown cocarcinogenic and irritant activity in cell and mouse models and have caused gastric and pulmonary problems in animals grazing on plants containing these compounds. However, these compounds have also shown anti-HIV and anticancer activity. To determine if Palauans are ingesting diterpene esters in traditional preparations of *Phaleria nisidai*, simplexin, acetoxyhuratoxin and huratoxin were quantified from methanol and aqueous extracts prepared in the lab and in aqueous infusions prepared by six traditional healers in Palau. Diterpenes were not detected in traditional aqueous extracts prepared by healers in Palau, or in aqueous extracts prepared in our lab, but were detected in the methanol extract. PBMC proliferation as well as their production of IFNγ was measured and it was found that aqueous extracts induce both PBMC proliferation as well as an increase in IFNγ production, although these effects were milder and significantly less than the activity demonstrated by the methanol extract. This further validated the traditional use of *P. nisidai* as an immunestimulating adaptogen and allayed concerns about public health issues with chronic ingestion of aqueous *P. nisidai* infusions.

Levels of simplexin, acetoxyhuratoxin, huratoxin and a bioactive xanthone, mangiferin, were analyzed in leaf samples from 227 trees collected from 92 populations of *Phaleria nisidai*. All Rock Island populations contained minute daphnane concentrations and lower amounts of mangiferin than populations on the largest Palauan island, Babeldaob. Savannah, scrub savanna and mature forest habitats all contained populations with exceptionally high levels of daphnanes, while the mangiferin content across habitats was more homogenous. Analysis of these
compounds by geographic variables can be useful to identify high-yielding chemotypes for biomedical or toxicological studies.

These studies demonstrate that Palauan traditional medicine can offer a source of plant metabolites with potent biological activity that corresponds to their traditional use. The discovery of simplexin and other diterpene esters in a plant consumed daily indicates that Palauans have found a way to selectively extract beneficial compounds while minimizing exposure to potentially harmful metabolites. The possibility also exists that Palauans have uncovered a low-dose therapeutic window at which diterpene esters can provide immunostimulant benefits while avoiding toxicological risks. The pharmacological potential of simplexin and related diterpenes in *Phaleria nisidai* should be reevaluated in light of these findings.
Acknowledgments

Undertaking a doctoral education allowed me to realize my dream of conducting ethnopharmacological research, meet amazing, brilliant people, and use cutting-edge equipment and software and I want to thank everyone involved in making that happen. Thanks to my advisor, Dr. Edward J. Kennelly, for providing support and patience while allowing me the time to figure things out and make and learn from mistakes in setting up experiments, time management and prioritizing lab work. I also want to thank him for providing an advanced phytochemistry lab to his graduate students, full of state-of-the-art instruments that we are lucky to have unfettered access to every day. Finally I am grateful to Dr. Kennelly for helping to connect me with Dr. Guido Pauli for a postdoctoral opportunity at University of Illinois-Chicago.

I am also extremely grateful for the co-mentorship of Dr. Michael J. Balick, who has been extremely instrumental in helping to formulate this dissertation project. I vividly remember when 18 months into my graduate studies we worked at the whiteboard at the Institute of Economic Botany literally drawing out the plans for this dissertation. This project would not have been accomplished without his facilitation of fieldwork in Palau and his previous establishment of research connections there built on his years spent developing trust cultivating respectful relationships among the people of Palau. The work he and Christopher Kitalong had previously conducted to negotiate a Materials Transfer Agreement protecting the intellectual property rights of Palauans allowed this work to proceed within a relationship built on mutual trust and understanding. Dr. Balick's concern for the intellectual property rights of cultures practicing traditional medicine is a model I will follow throughout my career.

None of this dissertation work would have possible without the help of many Palauans. My original contact in Palau was Christopher Kitalong, who initially introduced me to Phaleria
nisidai, the country of Palau, and their ethnomedicinal system. He also provided the raw botanical which I used for initial phytochemical and biological studies. Ann Hillmann and Obak Kitalong provided housing, transportation and numerous connections that I used throughout my fieldwork. I felt lucky to be included as a part of Ann, Obak, Chris, Mareva and Adangel's family while in Palau, and received so much support, warmth, comfort and wisdom from them. The Palau Bureau of Arts and Culture approved my interviews and I want to thank Ms. Kelly Marsh at this office for providing a place for this ethnographic data to be preserved in Palau. I also want to thank all state offices for acknowledging this permit and allowing me to conduct research within their borders.

The Belau National Museum provided plenty of support infrastructure, and I want to thank their director and curator, Ms. Pia Morei for her trust, support and resources and also for being the first research participant interviewed for this project. The most important resource Ms. Morei provided was a field guide, Mr. Van-Ray Tadao. I want to say sulang (thank you) to Van-Ray, half of the trees collected for this dissertation would not have been located without his intuitive sense for finding delal a kar and I want to thank him for hours spent accompanying me into the field, translating during interviews and assuring Palauans, on my behalf, that their rights to this knowledge will be respected.

I have to thank Ms. Mariana Sokau for selflessly volunteering her time to help me search for "the medicine." These trips allowed me to tap into her vast wealth of knowledge about Phaleria nisidai and many other Palauan medicinal plants, and for this I am extremely grateful. Mr. Yamason Sokau, thank you for collecting leaves from all of the trees I was too afraid to climb; thank you for helping prepare herbarium samples; and thank you for being a great field
guide on rock island trips. Your enthusiasm for research was infectious and inspiring. *Kom kmal mesulang* (thank you very much) to everyone that was a part of this project in Palau.

**All of the work presented in this dissertation was informed by the following healers,** who I would like to thank for their trust and generosity in sharing what they and their families know of *delalakar*: Susanna Dacheblai, Petrolina Santos, Ungilreng Takaus, Kalista Iyechad, Brenges Delkuu, Kliu Yuri, Sanae Mekui, Elliott Takeshi, Floris Tebelak, Tadao Skebong, Mariana Sokau, Justina Seualadaob, Apollonia Ngirchechol, Dibech Ellis, Lawrence Kitalong, Isabella Florencio, Wes Adelkeroi, Keradel Ngiraikelau, Chioko Kenty, David Ngarakesau, Henry, Rengiil Medalarak, Kannie Matsutaro, Roman Redep, Evence Kebekol and Pia Morei.

Dr. Dwight Kincaid has been a steady source of advice, both for this project and for problems encountered during graduate studies. When I had issues making sense of all of these numbers, trying to determine the best presentation of figures or statistical representation of reality, he has been there.

All of the bioactivity data could not have been gathered without the support of Dr. Jianda Yuan and the Memorial Sloan-Kettering Cancer Center. From helping to develop the experimental approach, assigning research assistants to help run assays and prepare blood samples to eventually trusting me to perform experiments in his lab when they could not be conducted at Lehman College, he has been an integral part of this dissertation.

Committee member Dr. Laurel Eckhardt has also provided invaluable input in helping me to understand some of the enormous complexity of the immune system, its connections with cancer and the reasons behind some of the assays conducted for this work and in the literature I have consulted.
I would also like to thank my past and current colleagues at CUNY, NYBG and MSKCC; including Drs. Adam Kavalier, Mario Figueroa, Chunhui Ma, Gema Flores, Keyvan Dastmalchi, James Lyles, Ulyana Munoz-Acuna, Shibiao Wu, Ricardo Kriebel, Rachel Meyer, Jillian De Gezelle, Lisa Offringa, Mu Zheng, Zhiwan Dong and Mr. Adam Negrin, Ms. Vanya Petrova, Ms. Lijuan Ma, Ms. Katherine Herrera, Ms. Teresa Rasalan, and Mr. Ian Cole for support, encouragement, passing along skills and wisdom, and helping with experiments. A special thank you is due to both Mr. Taylan Morcol and Mr. William Borenzweig, who have not only helped me to prepare samples, extractions and bioassays, but have also shared their infectious enthusiasm in working on this project. Thank you to Ms. Dolores Vitanza, Ms. Trish Carver and Ms. Michelle Meesawan for all of your assistance throughout the years and for helping to create a warm and comfortable environment at the Department of Biological Sciences and Institute of Economic Botany offices.

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I would have been unable to conduct this work without the support, interest and love of my parents, Nora and Michael Kulakowski, who have helped cultivate my interest in the natural world as a child to finally seeing me through to graduate school. I need to thank my sister, Kelly Kulakowski for providing a source of distraction and fun, and making me feel like a kid again when I visited her at home or school. I am grateful to my friends and former roommates Bryce Beloff, Emily Meyer and Margaret Mastrogiacomo for living with and tolerating the complicated schedule, habits and interests of a graduate student. My girlfriend and best friend, Stephanie
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<th>Description</th>
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<tbody>
<tr>
<td>ANG</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<td>bare/urban</td>
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<td>CTLA-4</td>
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<td>ELISA</td>
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<td>electrospray ionization</td>
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<td>Koror (island in Palau)</td>
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<td>limit of detection</td>
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<td>limit of quantification</td>
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<td>lipopolysaccharide</td>
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<td>mature limestone forest</td>
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<td>Malakal (island in Palau)</td>
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<td>mass spectrometry</td>
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<tr>
<td>NK</td>
<td>natural killer cell</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>nuclear magnetic resonance</td>
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<td>PBMC</td>
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<td>PHRCM</td>
<td>Palau Primary Health Care Manual</td>
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<td>RSD</td>
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<td>sandy atoll (representative of Kayangel)</td>
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<td>savanna</td>
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<td>scrub savanna</td>
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<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>KAY</td>
<td>Kayangel (island in Palau)</td>
</tr>
<tr>
<td>KR</td>
<td>Koror (island in Palau)</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantification</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Man</td>
<td>mangrove</td>
</tr>
<tr>
<td>MF</td>
<td>mature forest</td>
</tr>
<tr>
<td>MLF</td>
<td>mature limestone forest</td>
</tr>
<tr>
<td>MLK</td>
<td>Malakal (island in Palau)</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSKCC</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MY</td>
<td>Meyuns (island in Palau)</td>
</tr>
<tr>
<td>NC</td>
<td>negative control</td>
</tr>
<tr>
<td>NGK</td>
<td>Ngeruktabel (rock island in Palau)</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NYBG</td>
<td>New York Botanical Garden</td>
</tr>
<tr>
<td>ONG</td>
<td>Ongael (rock island in Palau)</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PHRCM</td>
<td>Palau Primary Health Care Manual</td>
</tr>
<tr>
<td>RI3</td>
<td>rock island 3' (rock island in Palau)</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation (see CV)</td>
</tr>
<tr>
<td>Sand</td>
<td>sandy atoll (representative of Kayangel)</td>
</tr>
<tr>
<td>Sav</td>
<td>savanna</td>
</tr>
<tr>
<td>ScSav</td>
<td>scrub savanna</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
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<td>Kayangel (island in Palau)</td>
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<td>mature limestone forest</td>
</tr>
<tr>
<td>MLK</td>
<td>Malakal (island in Palau)</td>
</tr>
</tbody>
</table>
Chapter 1

Ethnopharmacology, chemistry and ecology of *Phaleria nisidai* in the Republic of Palau

1. Palau

Palau is a country comprised of more than 586 islands, occupying 459 sq km of land, and home to just over 20,000 inhabitants (Central Intelligence Agency, 2014). Palau is located 800 km E of the Philippines and 800 km NNW of Papua New Guinea, and its islands make up the westernmost islands of Micronesia. The vast majority of Palauan islands are the 'Rock Islands,' exposed limestone coral ranging in size from just a few square meters to the largest Rock Island, Ngeruktable, at 18.4 sq km. Other large islands in Palau include Babeldaob and Koror, which are volcanic in origin. Only 12 islands are continuously inhabited (Office of Environmental Response and Coordination, 2002) with over 80% of the population living in Koror and southern Babeldaob (Central Intelligence Agency, 2014). Babeldaob is the largest island in Palau (372 sq km) and contains the highest point in the country, Mount Ngerelchelchuus at 234 m.

Micronesia has been named a "Top 25 Biodiversity Hotspot" (Myers et al., 2000), and Palau is Micronesia's most biodiverse country (Hinchley et al., 2007). Terrestrial habitats in Palau host 109 endemic plant species out of 1260 total plant species (Office of Environmental Response and Coordination, 2002).

2. Traditional medicine in Palau

Humans have inhabited Palau for at least 3500 years (Canfield, 1980) and have spent the majority of this time surviving off of the natural resources existing here. Foods, shelter, forms of transportation, clothing, decoration and medicine were all extracted from the land and sea blanketing and surrounding the islands. Early migrants brought plants from their former homes
and also found new plants in their new home of Palau, so the traditional knowledge being studied today represents an ethnobotanical culture greater than 3500 years old. Some of these old folkways are still being practiced in Palau and include fishing methods and fisheries management practices, canoe making and *bai* (traditional male meeting house) building. Traditional medicine is also still being practiced in Palau. There is only one hospital in the country, with a few clinics and infirmaries operating in local villages with limited hours and limited pharmaceutical supplies. Therefore, traditional medicines, although often preferred to conventional medicines, may sometimes be used out of necessity, if not out of preference.

The ethnomedicinal data that initially informed this work was collected by other from 2007 to 2011 as part of the Plants and People of Micronesia/Biodiversity and Human Health Program, an initiative that included researchers and physicians from the Institute of Economic Botany at the NYBG, the Beth Israel Continuum Center for Health and Healing, the Belau National Museum and the Republic of Palau Ministry of Health. Some of this data was published as part of the Palau Primary Health Care Manual: Health Care in Palau: Combining Conventional Treatments and Traditional Uses of Plants for Health and Healing (PPHCM) (Dahmer et al., 2012). This book is a collection of local names, botanical description, range, traditional uses, recipes for preparation and pharmacological and toxicological properties of eighty-nine plants used in Palauan medicinal preparations. It is a reference for medical professionals working in Palau, but also for traditional healers and laypeople to better understand common medical conditions in the country as well as the pharmacological and toxicological properties of the plants they are using for medicine. This book carries on a tradition of ethnomedicinal research in Palau, began in the early 1940s by Japanese investigators such as Masayoshi Okabe (DeFilipps et al., 1988).
Many Palauans are concerned that their traditional knowledge is being diluted by conventional practices. Older Palauans are seeing a rapid rise in indifference towards traditional practices by the youth brought about by modernization trends that have been taking place in the country since World War II, as discussed by Stevenson Kuartei, Minister of Health (Dahmer et al., 2012). The PPHCM and associated research, including this project, serve to document and preserve traditional plant knowledge before it is lost.

In research conducted by our collaborators for the Plants and People of Micronesia/Biodiversity and Human Health Program, Palauans indicated that one of the most widely-used and revered plant medicines in Palau was Phaleria nisidai Kanek. This plant is known as delalakar in Paluan, which translates to 'Mother of medicine' in English.

Figure 1.1 Panel A: Phaleria nisidai with shrubby habit, showing alternate leaf orientation and prominent venation. Panel B: Fruit and flowers, note cauliforous inflorescence born on stem or terminal axils.
3. Description and taxonomy of Phaleria nisidai

*Phaleria nisidai* is a shrub to small tree, up to 15m tall, with opposite leaves, 15-20 cm long. It has a white cylindric floral tube, about 20-35 mm long, with 4-5 calyx lobes. The one-seeded fruit is a drupe, globose and red at maturity (Figure 1.1) (Kanehira, 1933). This tree has a range from Southeastern Asia and Sri Lanka through Malaysia, to Micronesia, Northern Australia eastward to Samoa and Tonga (Smith, 1979). In Palau it was reported to grow in volcanic lowland forest and freshwater swamp forest (Kitalong, et al., 2008).

*Phaleria nisidai* is a member of the family Thymelaeaceae. Along with Euphorbiaceae, the Thymelaeaceae is the only other plant family known to contain daphnane- and tigliane-type diterpene esters (Borris et al., 1988). Its most well-known genera include *Daphne* and *Pimelea*. *Daphne* includes poisonous, ornamental plants common to temperate regions of the world; such as *D. mezereum*, one of the oldest toxic plants known, with reference to its toxicity made by Dioscorides (Kingsbury, 1964). Around 80 species of *Pimelea* grow in Australia (Pettit et al., 1983) and this genus contains many poisonous species responsible for the development of St. George disease in grazing animals in Australia (see Chapter 3 for further discussion of *Pimelea* toxicity).

4. Traditional uses of Phaleria nisidai

Ethnographic data collected for the PPHCM indicate that *Phaleria nisidai* is used as medicine for headaches and menstruation, for overall strengthening and as an energizing tonic. It is also mentioned as being used in combination with eight other plants to cleanse the body of unhealthy things (Dahmer et al., 2012). This use pattern indicated that Palauans are using *P. nisidai* as an adaptogen or immunostimulant. This preliminary data on *P. nisidai* was gathered as
part of broad surveys in which healers would list the names and uses of many medicinal plants. A more specific ethnobotanical survey was conducted as part of this dissertation research. Structured interviews were conducted with twenty six Palauan healers focusing only on Phaleria nisidai. Uses, preparation methods, doses, collection site, and contraindications were recorded in addition to some demographic data about the interview subject. The questionnaire used is shown in Appendix A.

A total of 105 individual use reports were generated, averaging about four unique uses per person. Each use report is listed and tallied in Table 1.1. A word cloud was constructed from interview results and is a way to represent frequency of words in a text. In this case it was used to measure the frequency of use mentions from these twenty-six interviews and can be found in Figure 1.2. More frequently mentioned uses appear as larger words in the cloud. Besides ‘system cleaner’ other frequently mentioned uses are for energy, prevention, strength, flu, to treat common sickness, diabetes and high blood pressure. Wordle software was used to construct this word cloud (Feinberg, 2008).

Use as a system cleaner, for prevention, to treat common sickness, for building strength, for energy are all non-specific indications for this plant. Non-specific uses for a remedy indicate that the medicine may be an adaptogen, acting on the immune system; which has both non-specific (innate) and specific (adaptive) components (Murphy et al., 2007) which interact together to exert effects throughout the body. Over 57% of healers used Phaleria nisidai as a system cleaner, 27% used this beverage to increase energy, 23% used it to treat the flu. Almost 20% of participants used it to keep away sickness and 15% used it to increase strength and/or treat common sickness. The immunomodulatory uses of P. nisidai, in conjunction with its reports of being used to both increase strength and increase energy confirm the initial hypothesis that
this plant is used as an adaptogen. With an average of four unique uses per person this plant is used to treat conditions in many physiological systems throughout the body; including immune, cardiovascular, and digestive systems.

When 'system cleaner' was mentioned as a use it was mostly accompanied by a specific type of cleansing: to flush toxins, cleanse organs after birth of first child, eliminate viruses or bacteria, during menstruation, for bladder or intestinal problems and ameliorate food poisoning. 'System cleaner' does not usually mean a diuretic or purgative, as it was only mentioned once in conjunction with food poisoning and once to clean the bladder/intestines. In most other cases we believe 'system cleaner' means an agent to help enhance clearance of toxins from the blood or liver or to stimulate the immune system to clear bacteria and viruses from the body. Macrophages are a component of the immune system that cleanse the system through phagocytosis and destruction of pathogenic organisms (Beutler, 2004) and this plant may manifest activity as a 'system cleaner' by activating these immune cells (Matsuda et al., 2005a).
Figure 1.2 Word cloud representing uses for Phaleria nisidai. Size of use corresponds to frequency of mention in twenty-six targeted structured interviews for this plant.
Table 1.1 Use reports for *Phaleria nisidai* from twenty-six study participants. Number of reports and % of healers mentioning each use are displayed.

<table>
<thead>
<tr>
<th>Use</th>
<th># Reports</th>
<th>% Users</th>
<th>Use</th>
<th># Reports</th>
<th>% Users</th>
</tr>
</thead>
<tbody>
<tr>
<td>system cleaner</td>
<td>15</td>
<td>57.7</td>
<td>liver cleanse/problems</td>
<td>2</td>
<td>7.7</td>
</tr>
<tr>
<td>energy drink</td>
<td>7</td>
<td>27.0</td>
<td>lactation</td>
<td>2</td>
<td>7.7</td>
</tr>
<tr>
<td>diabetes</td>
<td>6</td>
<td>23.1</td>
<td>make other medicine</td>
<td>2</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>work better</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flu</td>
<td>6</td>
<td>23.1</td>
<td>runny nose</td>
<td>2</td>
<td>7.7</td>
</tr>
<tr>
<td>high blood pressure</td>
<td>6</td>
<td>23.1</td>
<td>abortion</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>keep away sickness, avoid hospital, live long</td>
<td>5</td>
<td>19.2</td>
<td>bodyache/soreness</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>constipation</td>
<td>4</td>
<td>15.4</td>
<td>cholesterol</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>strength</td>
<td>4</td>
<td>15.4</td>
<td>cold</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>treat common sickness (not prevention)</td>
<td>4</td>
<td>15.4</td>
<td>cough</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>fertility/to get pregnant</td>
<td>3</td>
<td>11.5</td>
<td>diet medicine</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>heart disease</td>
<td>3</td>
<td>11.5</td>
<td>feel better</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>migraine/headache</td>
<td>3</td>
<td>11.5</td>
<td>feel lighter</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>stomachache, including 1 associated with menstruation)</td>
<td>3</td>
<td>11.5</td>
<td>gout</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>appetite</td>
<td>2</td>
<td>7.7</td>
<td>hangover</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>cancer</td>
<td>2</td>
<td>7.7</td>
<td>heart feels weird/pumps too strong</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>diarrhea</td>
<td>2</td>
<td>7.7</td>
<td>kidney stone</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>fever</td>
<td>2</td>
<td>7.7</td>
<td>stings (fish)</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>hydration</td>
<td>2</td>
<td>7.7</td>
<td>toothache</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>immune booster</td>
<td>2</td>
<td>7.7</td>
<td>venereal disease</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>TOTAL</td>
<td><strong>105</strong></td>
<td><strong>NA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. *Phaleria nisidai* as combination medicine

*Phaleria nisidai* is often used in combination with other plants. In interviews Palauans indicated that one must clean the system of any bacteria and virus before ephemeral symptoms or long-term damages can be ameliorated. In this work, and in previous reports (Dahmer et al., 2012), *P. nisidai* was a component of many preparation, acting to cleanse the system before other plants in the mixture could fix the more overt manifestations of the disease.
Seventeen healers use *Phaleria nisidai* by itself, while other informants use it only in combination with other plants. Depending on the use or severity of illness, some healers that use *P. nisidai* by itself also have a use for it in combination with other plants. Two common formulations were encountered during this ethnobotanical survey (Figure 1.3). Combination A was a mixture of *P. nisidai* with *kirrai* (*Scaevola taccada* (Gaertn.) Roxb., Goodeniaceae) and *kelsechedui* (*Vitex trifolia* L. var. *trifolia*, Lamiaceae). Sometimes *techellelachull* (*Cassytha filiformis* L., Lauraceae) and/or *ukelellachedib* (*Phyllanthus palauensis* Hosok., Phyllanthaceae) was added. Combination B was a mixture of only *Phaleria nisidai* and *ukelellachedib* (*Phylanthus palauensis*). Fifteen healers reported using Combination A and four healers reported
using Combination B. Table 1.2 shows the relevant pharmacological activity of these plants as well as the number of users reporting using these plants with *P. nisidai*.

**Table 1.2** Plants used in common combination recipes with *Phaleria nisidai*. Number of users combining each plant with *Phaleria nisidai* is also noted.

<table>
<thead>
<tr>
<th>Local and scientific name</th>
<th># Users</th>
<th>Relevant Pharmacological Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COMBINATION A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>kirraikorrai</em></td>
<td>15/26</td>
<td>No pharmacological reports found.</td>
</tr>
<tr>
<td><em>Scaevola taccada</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goodeniaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>kelsechedui</em></td>
<td>15/26</td>
<td>Aqueous extract decreased LPS-induced IL1β &amp; IL6; increased IL10 in RAW 264.7 macrophages (Matsui et al., 2009). Aqueous extract decreased LPS-induced inflammatory chemokines (CCL3, CXC-L10 &amp; COX-2) and NF-κB cytokine in RAW 264.7 macrophages (Matsui et al., 2012).</td>
</tr>
<tr>
<td><em>Vitex trifolia</em> var. trifolia*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamiaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>techellelachull</em></td>
<td>5/26</td>
<td>Alkaloid-enriched extract and isolated aporphine alkaloids cytotoxic to HeLa cells and antiparasitic to <em>Trypanosoma brucei brucei</em> (Hoet et al., 2004). Isolated alkaloids and flavonoids had vasorelaxant properties on rat aortic preparations in vitro (Tsai et al., 2008).</td>
</tr>
<tr>
<td><em>Cassyytha filiformis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>COMBINATION B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ukeellachedib</em></td>
<td>7/26</td>
<td>Aqueous extract of <em>Phyllanthus niruri</em> increased expression of surface activation marker CD69 increased proliferation of T- and B-lymphocytes and increased IL4 and IFNγ production by splenocytes and phagocytosis and TNFα release by macrophages (C S Nworu et al., 2010). Aqueous extract of <em>P. niruri</em> increased expression of MHC-II and activation markers and activation and costimulation markers of dendritic cells (DCs), causing DCs to more efficiently present antigen (Chukwuemeka S Nworu et al., 2010). Methanol and aqueous extracts of <em>Phyllanthus simplex</em> showed hypoglycemic effects in normal rats and antihyperglycemic effects in diabetic rats (Shabeer et al., 2009).</td>
</tr>
<tr>
<td><em>Phyllanthus palauensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phylanthaceae</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. **Knowledge patterns of Phaleria nisidai**

The number of use reports for *Phaleria nisidai* was quantified for each informant. The most uses one person reported was six and the lowest amount of uses was one. Some demographic data was collected for each informant, such as age and residence. Females (n=15)
knew on average 4.1 uses, while males (n=11) knew on average 3 uses (Figure 1.4, Panel A). ANOVA was performed, followed by the Tukey HSD test for significance, to determine differences in number of uses known by gender. Base R software packages (R Core Team, 2013) were used for these analyses. The p-value was insignificant (0.072), but close to significance. A power analysis (power=0.8) was performed, using the ‘pwr’ package (Champely, 2012) revealing that if the same trend in results continued, a total of 109 participants would need to be interviewed to achieve statistical significance.

**Figure 1.4** Number of uses reported per person by demographics. Panel A: number of uses by gender. Panel B: number of uses by migration status (yes: participant migrated from state of birth; no: participant did not migrate). Panel C: number of uses by residence in Koror and Babeldaoab vs. outer islands (Kayangel, Angaur, Peleliu).

A question asked of every informant was "Do you live in the same place you were born?" This question was included to infer ties the informant had to his or her relatives. We believed that a person who did not migrate from their state of birth would be more likely to rely on traditional medicine because they had learned to be sustained by what is available in their village. We found that there was no significant difference (p=0.55) in number of uses known
between participants who have relocated (n=6, mean = 3.8) versus participants who have not left their state of birth (n=16, mean = 3.4) (Figure 1.4, Panel B). Although not statistically significant, comparing the means hints that informants who have relocated know slightly more uses than those who have not. This may indicate that healers integrate uses from their new place of residence when migrating. Many more observations are needed to confirm this hypothesis.

Babeldaob and Koror make up the main island chain of Palau. The islands of Koror are connected by a series of bridges and causeways to one another and to Babeldaob. The Belau National Hospital and the International Airport are on these islands and Koror is the commercial center of the country; including two supermarkets and several medical clinics. The capitol, Melekeok, is located on Babeldaob. These islands can be contrasted to the only other populated islands of Palau; Kayangel, Peleliu and Angaur (not including the Southwest Islands, 600 km from the main islands). These islands can only be accessed by ferry (and the occasional aircraft). There is a clinic on each island but the hours and supply of pharmaceuticals are limited. Many Palauans living on these "outer islands" do not frequent Koror or Babeldaob. We hypothesized that residents of outer islands know more uses for Phaleria nisidai because they are more isolated from conventional medicines available at supermarkets, hospitals and doctor's offices than those living in Koror and Babeldaob and would have to rely more on this medicinal plant. Residents of Koror and Babeldaob knew the same amount of uses (n=17, mean=3.59) as residents of the "outer islands" (n=9, mean=3.67).

No significant difference was observed by grouping these twenty-six participants by gender, migration status, or main vs. outer island residency. The number of uses known was constant for each of these grouping categories. These findings may indicate that knowledge of
Phaleria nisidai is an integral part of Palauan culture and levels of knowledge about this plant are not altered by residence, gender, or migration pattern.

7. Dissemination of Phaleria nisidai knowledge

Participants were asked "who has taught you how to prepare and use delal a kar?" In most cases a grandparent or parent taught about Phaleria nisidai. Four participants learned of this plant from following the Modekngei religion; a combination of traditional Palauan customs and Christianity. In one instance a woman was chosen by a male healer as an apprentice.

Figure 1.5 Knowledge transfer by gender. Number of healers from each gender taught about Phaleria nisidai by a male, female or both (relatives, grandparents, parents or church).

To determine if gender played a role in the transfer of Phaleria nisidai knowledge, the gender of the teacher was plotted for male and female users (Figure 1.5). The majority of males (5/8, 62.5%) were taught by both males and females. In these cases, the response to "Who has
taught you how to prepare and use *delal a kar*?" was parents, grandparents, the church or the community. Only 1/8 males were taught by another male and 2/8 (25%) of males were taught by a female. Females taught 57% (8/14) of female users, males taught 3/14 (21.4%) females and 3/14 female users were taught by both men and women. This data was entered into a contingency table and a chi-square test was performed. Following a chi-squared test for trend this pattern was not significant ($p=0.154$). A power analysis using the 'pwr' package in R (Champely, 2012) for this chi-squared test (moderate effect size = 0.3, power = 0.8, significance leve = 0.05) determined that data from 107 interviews would have made these results significant. There may be a high prevalence of female-female knowledge transfer because *P. nisidai* is a component of many first birth hotbath ceremonies (Dahmer et al., 2012). Informal observations during fieldwork in Palau revealed that only women are involved in harvesting plants for hotbath ceremonies, and this may be the first exposure young girls have with the collection and preparation of medicinal plants.

The twenty-six interviews conducted for this work did not result in findings of statistical significance. Two separate power analyses of these results (for the data in Figure 1.4, Panel B and Figure 1.5) indicated that a sample size of around 110 interviews would have provided enough data to obtain significant results based on the questions about *Phaleria nisidai* uses and knowledge transfer that were asked. The interviews already conducted should be looked at as a pilot study, informing future ethnobotanical surveys on *P. nisidai* in Palau.

8. Reported side effects of *Phaleria nisidai*

Most participants did not report any side effects (Table 1.3) with *Phaleria nisidai* usage. One major contraindication stated is that its use should be avoided in pregnancy. Two people reported diarrhea as a side effect. One participant reported that this medicine "can kill some
people if they drink too much." While alarming, further details were not provided. Many people drink *P. nisidai* tea every day, or as often as needed, with one family reporting that it is consumed in place of water. Only a few participants used *P. nisidai* sporadically, worrying that if it is used more than a few times per year it could lead to unspecified complications.

<table>
<thead>
<tr>
<th>side effects and contraindications</th>
<th>responses</th>
<th>use frequency</th>
<th>responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>no side effects</td>
<td>6</td>
<td>everyday</td>
<td>12</td>
</tr>
<tr>
<td>don't drink when pregnant</td>
<td>5</td>
<td>whenever needed</td>
<td>4</td>
</tr>
<tr>
<td>diarrhea</td>
<td>2</td>
<td>almost every day</td>
<td>3</td>
</tr>
<tr>
<td>too much will decrease insulin</td>
<td>1</td>
<td>2-3 times per week</td>
<td>2</td>
</tr>
<tr>
<td>don't drink if trying to get pregnant</td>
<td>1</td>
<td>throughout the day for one</td>
<td>1</td>
</tr>
<tr>
<td>weight loss</td>
<td>1</td>
<td>day every other week</td>
<td></td>
</tr>
<tr>
<td>don't eat with fruits</td>
<td>1</td>
<td>7 days straight, 2-4 times</td>
<td>1</td>
</tr>
<tr>
<td>make women bleed more</td>
<td>1</td>
<td>a year</td>
<td></td>
</tr>
<tr>
<td>unspecific side effects if you drink too much</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mild dizziness with &quot;overdose&quot;</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>can kill some people</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. **Adaptogens and botanical immunomodulators**

Adaptogens are a class of medicines taken to increase the body’s resistance to stressors from various sources, exerting a normalizing influence on the body’s physiological processes (Brekhman and Dardymov, 1969). Something is considered an adaptogen if its "effect is non-specific in that the adaptogen increases resistance to a very broad spectrum of harmful factors ("stressors") of different physical, chemical and biological natures" (Panossian et al., 1999). Evidence indicates that many adaptogenic herbs act through immunomodulatory mechanisms, such as the Indian Ayurvedic Rasayana herbs *Tinospora cordifolia, Asparagus racemosus, Withania somnifera, Piper longum, Terminalia chebula, and Emblica officinalis* (Rege et al., 1999) and ginseng (*Panax ginseng*) from Traditional Chinese medicine (Chang et al., 2003). A
treatment that stimulates the immune system can improve resistance to a number of pathogens, seeming to act in a non-specific manner because the innate immune system is non-specific and the adaptive, antigen-specific, immune system can respond to many kinds of challenges.

Immunostimulants are often employed, with or without oncologist's recommendations, in the treatment, prevention or symptom-mitigation of cancer (Leung and Fong, 2007). Due to the multiple sources of stress cancer patients endure, from radiation and/or chemotherapy to impact of the tumor itself, it is believed that adaptogens can improve the overall wellness of cancer patients and even contribute to the fight against cancer (Chang et al., 2003). Many naturally occurring plant compounds, including Taxol®, vincristine, camptothecin, doxorubicin and podophyllin have been used as chemotherapeutic agents, acting directly on tumor cells, but botanicals can also act as immunotherapeutic agents in cancer. These products may act by improving the response to cancer vaccine and/or counteracting immunosuppression caused the tumor itself or radiation/chemotherapy (Oldendick et al., 2000).

10. Immunotherapy and cancer

The study of cancer immunotherapy provides a good illustration of how immunomodulatory treatments work. The use of complementary and alternative medicine (CAM), including herbal medicines, in patients with cancer is on the rise (Oldendick et al., 2000). One of the main reasons cancer patients use herbal medicines is to protect and stimulate the immune system and increase quality of life (Oldendick et al., 2000). Advances in the understanding of the interactions between the immune system and tumor cells have helped to make great strides in field of immunotherapy (Hurwitz and Gabrilovich, 2008).

The host immune system possesses the ability to recognize, control, and even eliminate tumors, just as it would treat a foreign pathogen; however tumors display immunosuppressive
strategies which aid in escape (Ribas et al., 2003). Therapies, including vaccines, have been
developed to take advantage of, and amplify, the ability of the host’s intrinsic immune
mechanisms to combat cancer and overcome tumor-induced immunosuppression. For example,
our colleagues at the Immune Monitoring Facility or Memorial Sloan-Kettering Cancer Center
(MSKCC) have analyzed immune markers in patients receiving ipilimumab, an anti-CTLA4
(cytotoxic T-lymphocyte antigen-4) antibody, as treatment for melanoma, which was approved
by the FDA in 2011 (Acharya and Jeter, 2013; Yuan et al., 2011). CTLA-4 (cytotoxic T-
lymphocyte angiten-4) is a costimulatory receptor on the surface of T-cells and its binding by
antigens such as B7-1 on antigen-presenting cells (APCs) and other T-cells results in
downregulation of the T cell response and is responsible for immunosuppressive effects tumor
cells exert on the host immune system (Page et al., 2010).

In order for an immune cell to become activated it must be presented with an antigen
(from a tumor, pathogen, or vaccine) in the appropriate microenvironment, which is determined
by the cytokines and chemokines released and co-stimulatory molecules expressed on the
involved cells (Ribas et al., 2003). Tumors have the ability to downregulate immune mechanisms
by decreasing both cytokine output and expression of co-stimulatory molecules; thus in cancer
immune cells often do not receive the appropriate immune activating signals (Gyorki et al., 2013;
Ribas et al., 2003). Vaccine adjuvants and general immunostimulators are two types of therapies
that can induce the appropriate microenvironment in cancer. Adjuvants act to stimulate the host
immune response to a vaccine and general immunostimulators increase host sensitivity and
overcome immunosuppressive strategies. Gamma-interferon (IFN-γ) is a key cytokine
responsible for generating this microenvironment (Murphy et al., 2007; Schroder et al., 2004).
IFN-γ, tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) are all cytokines released by T cells to initiate and maintain immunostimulation (Murphy et al., 2007). IL-2 stimulates the growth, selection, and survival of antigen-specific cytotoxic T cells; TNF-α activates macrophages and induces nitric oxide (NO) production to destroy internalized pathogens; while IFN-γ activates macrophages and causes an increase in antigen presentation in macrophages and other cells (Murphy et al., 2007). An increase in these cytokines leads to an inflammatory microenvironment suitable for generation of an immune response to antigen (Murphy et al., 2007; Wang et al., 2007), either from the tumor itself or from an injected cancer vaccine.

Immunotherapy is also important because of the detrimental effects on immunity caused by chemotherapy and radiation, which reduce the body’s ability to fight not only cancer but also other diseases (Bodey, 1986). Many natural products have been shown to have both anticancer and immunostimulant activity including astragalus (*Astragalus propinquus* syn. *A. membranaceus*), *Coriolus versicolor*, shiitake mushroom (*Lentinula edodes*), beta-glucans derived from fungus (Patwardhan and Gautam, 2005), *Tinospora cordifolia* *Andrographis paniculata* (Leung and Fong, 2007) and curcumin (Kamat et al., 2007) and some have shown vaccine adjuvant activity, including QS-21 (Livingston et al., 1994), *Echinacea spp.*, *Curcuma longa* (turmeric), and maitake (*Grifola frondosa*) (Ragupathi et al., 2008).

11. Interferon-γ and immune monitoring

IFNγ is released by NK cells, T<sub>H</sub>1 helper cells, cytotoxic (CD8+) T cells and antigen-presenting cells (APCs) (Schroder et al., 2004; Tannenbaum and Hamilton, 2000) and can be used diagnostically to indicate immune cell activation and proliferation and show maintenance of an immune response (Yuan et al., 2011). Chapter 2 outlines findings completed as a result of
monitoring IFNγ output by PBMCs. This cytokine was analyzed while conducting bioactivity-guided fractionation of an exhaustive extract of *Phaleria nisidai*. This method combined results from cell culture experiments with chemometric analytical methods to identify immunostimulatory components of *P. nisidai*.

Biological effects of IFNγ include increased antigen recognition by CD8+ T cells, upregulation of costimulatory molecules on antigen-presenting cells (APCs) such as macrophages, inhibition of cellular proliferation, and increased tumor cell killing by macrophages (Figure 1.6) (Murphy et al., 2007; Schroder et al., 2004). It is part of a positive feedback loop that increases immune system sensitivity and antigenic response. Because tumor cells have the ability to suppress the immune system and decrease antigen presentation IFNγ is a critical cytokine to monitor in cancer immunotherapy (Tannenbaum and Hamilton, 2000). It is one of the critical biomarkers monitored by our collaborators at MSKCC as they assess a patient's response to immune therapies in clinical trials.
Figure 1.6 Effects of IFNγ on tumor and the immune system (Murphy et al., 2007).

T\textsubscript{H}1 helper T cells are closely associated with IFNγ, becoming activated by and producing this cytokine (Schroder et al., 2004) and many researchers have demonstrated that the induction of T\textsubscript{H}1-promoting cytokines, such as IFNγ by specific adjuvants, can enhance antitumor immunity and reduce or even prevent tumor growth (Patwardhan and Gautam, 2005).

12. Bioactivity of Phaleria nisidai

Despite the frequency of Phaleria nisidai use in Palau, only two research groups have reported on its bioactivity, with a total of four published articles between them. Two articles explore the immunomodulatory activity of this plant. Crude extracts from P. nisidai have been shown to inhibit the tumor-induced reduction of TNF-α, IL-2, and IFNγ cytokines in splenocytes isolated from carcinoma bearing mice and, in the case of IFNγ and IL-2, increase production
level above what is produced in a healthy state (Matsuda et al., 2005b). Macrophages treated with *P. nisidai* extract were shown to be more effective in inducing T cells to produce TNF-α, IL-2, and IFNγ cytokines and biactivity-guided fractionation revealed a mixture of acylglucosylsterols to be responsible for this activity (Matsuda et al., 2005a) (Figure 1.7). This is in parallel with the increase in phagocytic activity caused by a *P. nisidai* crude extract and purified acylglucosylsterol mixture on RAW 246.7 mouse leukemic macrophage cells observed in a related study (Matsuda et al., 2005a).

![Figure 1.7](image)

**Figure 1.7.** *In vivo* and *in vitro* effects of 50% ethanolic extract of *Phaleria nisidai* on cancer and immunity (Matsuda et al., 2005a, 2005b).

Matsuda's group has also investigated the antidiabetic effects of *Phaleria nisidai*. A 50% ethanolic extract exhibited blood glucose-level decreasing effects in diabetic obese-type KKAy mice and inhibited a blood glucose rise after an oral sucrose-loading test in healthy ddY mice. Bioactivity-guided fractionation led to the isolation of the xanthone mangiferin as the antidiabetic component of *P. nisidai* extracts (Matsuda et al., 2004). It should be noted that Matsuda's group states the name of the plant as *Phaleria cumingii* (Meisn.) F. Vill. According to
botanical experts assisting with this dissertation work, this is a mistaken identification of *Phaleria nisidai* Kaneh., the only species of this genus found in Palau (Kitalong et al., 2008). Another group has demonstrated the estrogenic activity of a benzophenone glycoside and its aglycone, isolated from *P. nisidai* (Kitalong et al., 2012).

These previous studies indicate that this plant may be effective as an immunostimulant and/or vaccine adjuvant in cancer treatment. Also the traditional use of this plant as an adaptogen may be therapeutic for the detrimental secondary syndromes of cancer patients, as chemotherapy and radiation contribute many stresses to the body and often cause immunodeficiency and chronic fatigue (Bodey, 1986; Rovigatti, 2012).

13. **Comprehensive review of Phaleria nisidai phytochemistry**

Investigations into the phytochemistry of *Phaleria nisidai* are rare, with the only compounds reported for this plant (Figure 1.8) coming from the bioactivity studies cited above. Leaves of this plant were found to contain the glycosylated xanthone mangiferin (1), a mixture of acylglucosylsterols (2), and the aliphatic acid tetracosanol (3) (Matsuda et al., 2005a, 2004). Another group isolated the benzophenone glycoside iriflophenone 2-α-rhamnopyranoside (4) and a flavonoid; genkwanin 5-β-D-primeveroside (5) (Kitalong et al., 2012a). Triterpene, sesquiterpene and benzophenone glycosides, in addition to mangiferin, have been identified in fruits of the related plant *Phaleria macrocarpa*, from Indonesia (Hakim et al., 2004; Kurnia et al., 2008; Oshimi et al., 2008).

14. **Endotoxins**

When conducting any assays involving the immune system one must be sure that samples are free of endotoxins. Endotoxins are components of the outer membrane of Gram-negative bacteria (Caroff et al., 2002) that are more correctly referred to as lipopolysaccharides (LPS).
Non-specific binding to toll-like receptors by LPS causes activation of innate immunity in mammals (Alexander and Rietschel, 2001; Caroff et al., 2002). In some studies of botanical immunomodulators LPS contamination caused the majority of monocyte/macrophage activation, and this activity was eliminated by removing LPS from the sample (Pugh et al., 2008). Wild-harvested leaves, such as those used in this dissertation work, are likely to house endotoxin-producing bacteria and methods used to partition, fraction and purify botanical samples may not sufficiently remove endotoxin to inactive levels for in vitro experiments (Pugh et al., 2008). Thus it is crucial to prepare botanical treatments in a clean, dust-free environments and have treatment samples tested for LPS before use in cell culture experiments.

![Compounds](image)

**Figure 1.8** Compounds identified from *Phaleria nisidai* (Kitalong et al., 2012a; Matsuda et al., 2005a, 2004). 1: mixture of acylglucosysterols where R₁ is either hydrogen or acetyl group and R₂ is a fatty acid (palmitoyl, oleoyl, α-linolenoyl, stearoyl, linoleoyl). 2: mangiferin. 3: tetracosanol. 4: iriflophenone 2-O-α-rhamnopyranoside. 5: genkwanin 5-O-β-D-primeveroside
15. Diterpenes

The Thymelaeaceae and Euphorbiaceae are the only two plant families known to contain daphnane- and tigliane-type diterpene esters (Blumberg, 1988; Borris et al., 1988; Liao et al., 2009). A review of the literature reveals that within the Thymelaeaceae all tigliane and daphnane diterpene esters are located in the Thymelaeoideae subfamily, which includes the following genera: *Phaleria, Gnidia, Pimelea, Daphne, Daphnopsis, Stellera*, and *Wikstroemia* (Borris et al., 1988). Diterpene esters have been reported in these other genera, but until this work, have never been observed in *Phaleria*.

Diterpene esters are notorious for their irritant and co-carcinogenic properties (Adolf et al., 1988; Hecker, 1985) and their activities are so potent and reproducible that some naturally derived diterpene esters are used to induce clinical cellular responses. TPA (12-0-tetradecanoylphorbol-13-acetate; also known as PMA, phorbol 12-myristate 13-acetate) is used as a promoter to stimulate *in vitro* cellular proliferation and tumor formation (Katiyar, 2005) and mezerein, a daphnane orthoester, is used to cause melanoma cells to terminally differentiate (Fisher, 2005). Prostratin is a non-promoting tigliane diterpene ester (Gustafson et al., 1992) that inhibits de novo HIV-1 infection while potently upregulating latent HIV-1 expression from viral reservoirs in T cells (Kulkosky et al., 2001). Prostratin is currently undergoing pre-clinical trials at the AIDS Research Alliance, with NIH support, for investigational new drug status (AIDS Research Alliance, 2013). Speaking in September 2013, Paul Wender, the Stanford chemist who is responsible for the synthesis of prostratin believes the drug could be in clinical trials by late 2015 (Heitz, 2013). Further discussion of diterpene ester bioactivities can be found in Chapters 2 and 3.
Tigliane and daphnane diterpene compounds are characterized by a tricyclic ring system, with tiglianes having an identical skeleton to daphnanes, with the addition of a cyclopropanol to the 6-membered ring (ring C) (Figure 1.9). Tiglianes are progenitors to daphnanes (He et al., 2002). Many daphnanes, especially in the Thymelaeaceae, are of the orthester type (He et al., 2002) and are unique in having three alkoxy groups from ring C linked to one carbon atom.

![Figure 1.9 Basic tigliane, daphnane and daphnane orthoester skeleta.](image)

16. Mangiferin

Xanthones are tricyclic polyphenols with various substituents located on rings A and B. Rings A and B are derived from the acetate (carbons 5-8) and shikimate (carbons 1-4) pathways, respectively. Mangiferin is a tetraoxygenated xanthone with a C-glucoside moiety (Figure 1.10). As reviewed by Vyas et al., (2012) it has potent antioxidant properties and anticancer potential linked to its antiinflammatory activity (Leiro et al., 2004; Pinto et al., 2005). Mangiferin also has a number of bioactivities relevant to the traditional uses of Phaleria nisidai (see Table 1.1).

Relating to its use as a stimulant and heart tonic it has been shown to act as a central nervous system (CNS)- and cardio-stimulant (Bhattacharya et al., 1972a), perhaps through inhibition of monoamine oxidase (Bhattacharya et al., 1972b). Mangiferin has shown anti-viral activity, acting against Herpes simplex through inhibition of HSV-1 and HSV-2 virion replication (Yoosook et
al., 2000) consistent with traditional use to clear viruses. Mangiferin also lowers blood glucose, both increasing insulin sensitivity (Ichiki et al., 1998) and decreasing insulin resistance (Miura et al., 2001). This activity is consistent with the anti-diabetic effects use of this plant in Palau. In contrast to the inflammatory diterpene esters, mangiferin exerts immunomodulatory function by decreasing inflammation, acting through inhibition of NF-κB pathway and Jun N-terminal kinase 1 (Leiro et al., 2004).

![Figure 1.10](image)

**Figure 1.10.** Basic xanthone showing acetate-derived ring A and shikimate-derived ring B.

17. Statistical Analysis and Boxplots

Statistical computations and graphs for this dissertation were prepared using the R language and software packages. Analysis of variance (ANOVA), tests for significance, such as Tukey HSD and correlation of vectors were performed using base and contributing R packages. In many cases throughout this work boxplots were used to compare variables. The boxplot (also known as a box and whisker plot) is a commonly recognized statistical graphic and shows the central tendency, range, outliers and middle 50% of the data points (Figure 1.11). The main box of a boxplot is specified by quartiles, where 25% of the data in a group is above the upper quartile (above the box), 25% of the data is below the lower quartile (below the box), and the interquartile range is the values between; comprising 50% of the observations (body of the box). The "whisker" represents the minimum or maximum values; however, if outliers are present the
whisker represents 1.5 times the interquartile range. Anything above or below this whisker is an outlier.

The boxplot was chosen to represent much of the data in this work because it shows the full range of values in a group, contrasted to bar graphs which only show the mean of a data set ± an error prediction. Boxplots can be especially useful in cases where a data set consists of many low variables with a handful of much higher values (see Chapter 4). Normally these outliers would greatly influence the mean, and a barplot would not show the variability in the data set, which can be important to the interpretation of phenomena.

Figure 1.11 Interpretation of boxplot details.
Chapter 2

Chemometric approach to identification of immunomodulatory compounds from *Phaleria nisidai* Kaneh., an adaptogenic tea from Micronesia.

1. Introduction

*Phaleria nisidai*, is known as *delalakar* in Palauan, which translates to 'Mother of medicine.' This plant has a history of use as an adaptogen; to invigorate, strengthen, heal and keep away sickness (See Chapter 1). Adaptogens are a class of medicines taken to increase the body’s resistance to stressors from various sources, exerting a normalizing influence on the body’s physiological processes (Brekhman and Dardymov, 1969). The Ayurvedic system has a special category of these medicines, called Rasayana. Evidence indicates that many adaptogenic herbs have immunostimulant activities, such as the Rasayana herbs *Tinospora cordifolia* (Willd.) Hook.f. & Thomson, *Asparagus racemosus* Willd., *Withania somnifera* (L.) Dunal, *Piper longum* Blume, *Terminalia chebula* Willd. ex. Flem., and *Emblica officinalis* Gaertn. (Rege et al., 1999) and ginseng (*Panax ginseng* C.A. Mey.) from the traditional Chinese medicine pharmacopeia (Chang et al., 2003). Table 1.1 and Figure 1.2 generated from ethnobotanical interviews in Palau indicate that this plant is used as a system cleaner, to prevent and treat common sickness and flu, as an immune booster and to generate energy and strength. These traditional uses align with the indications of an adaptogen, increasing "resistance to a very broad spectrum of harmful factors ("stressors") of different physical, chemical and biological natures" (Panossian et al., 1999).

Previous research has demonstrated immunostimulatory activity in leaf extracts of *Phaleria nisidai in vivo and in vitro* (Matsuda et al., 2005a, 2004). Specifically extracts and isolated compounds were shown to increase macrophage activity and production of pro-
inflammatory, immune-stimulating cytokines. In these studies *P. nisidai* was shown to enhance immune function, decrease tumor size and inhibit cell proliferation; and a mixture of acylglucosylsterols were determined to be responsible for activity. In addition to the acylglucosylsterols, mangiferin (Matsuda et al., 2004), the benzophenone iriflophenone-2-\(O-\alpha\)-rhamnoside and the flavonoid genkwanin-5-\(O-\beta\)-D-primeveroside (Kitalong et al., 2012) are the only compounds that have been identified from this plant so far.

In this study release of IFN\(\gamma\) by peripheral blood mononuclear cells (PBMCs) is monitored as a marker of immune stimulation during early-stage bioactivity-guided fractionation. This cytokine is released by NK cells, T\(_{H1}\) helper cells, cytotoxic (CD8+) T cells and antigen-presenting cells (APCs) and can be used diagnostically to indicate immune cell activation and proliferation and show maintenance of an immune response (Schroder et al., 2004; Tannenbaum and Hamilton, 2000). Its biological effects include increased antigen recognition by CD8+ T cells, upregulation of costimulatory molecules on macrophages, inhibition of cellular proliferation, and increased tumor cell killing by macrophages (Murphy et al., 2007). It is part of a positive feedback loop that increases immune system sensitivity and antigenic response. IFN\(\gamma\) has been monitored previously to measure immune stimulation caused by natural products in *in vitro* (Hong et al., 2005) and *in vivo* (Bani et al., 2006; Yamada et al., 2011) studies. We propose that *Phaleria nisidai*, a plant traditionally used as an immune stimulant will increase the production of IFN\(\gamma\), a major T cell-stimulating cytokine (Schoenborn and Wilson, 2007; Schroder et al., 2004), in PBMC culture.

In this study bioassay results are used to classify and group fractions according to immunostimulant activity. These fractions are simultaneously analyzed with a liquid chromatography-mass spectrometry-time of flight (LC-MS-TOF) instrument and data
characterizing chromatographic peaks (exact mass, retention time, ion intensity) is exported to MarkerLynx, a software package built on the Simca-P platform, used for analyzing multivariate metabolomics data. Orthogonalonal projection least squares-discriminatory analysis (OPLS-DA) is the multivariate approach used in this study because it is a supervised technique allowing the user to classify clusters in the scores plot as distinct groups; in this case, active fractions are one group and inactive fractions comprise the second group. An S-plot is generated which plots ions according to their importance in differentiating groups (Figure 2.1). This allows for tentative determination of compounds responsible for activity; and these compounds can be pulled from pure compound libraries or purchased from suppliers and tested individually, avoiding the need to isolate every compound in an active fraction as is traditionally done in bioactivity-guided fractionation and isolation. The experimental approach is summarized in Figure 2.2.

**Figure 2.1** Using an S-plot to discriminate markers corresponding to bioactivity.
Figure 2.2 Chemometric approach to bioactivity-guided fractionation.
2. Materials and Methods

2.1 Plant material

Samples of *Phaleria nisidai* were collected from five trees in Airai state, Ngetkib hamlet, Palau in July 2012 and grouped in bulk. Local expert and botanist Ann Hillman-Kitalong (Belau National Museum) performed sample identification and verification. Voucher specimens were prepared and deposited at the Belau National Museum, Natural History Section, specimen #DK035. Palauan intellectual property rights are reserved according to material transfer agreement docket #TCO-09B5012-MTA between the City University of New York and the Republic of Palau, active from December 31, 2008.

2.2 Extraction

Bulk *Phaleria nisidai* leaves collected as above were placed in an oven heated by a 30W incandescent bulb until dry (approximately 72 h) and shipped to New York, where the leaves were powdered in a plant mill. 400 g leaf powder was extracted in methanol under ultrasonic conditions for two hours at room temperature (3 x 4 L). Methanol extracts were combined and concentrated under reduced pressure, yielding 76 g for bioactivity testing and fractionation.

2.3 Pharmacological activity analysis

2.3.1 Preparation of cells

Buffy coat containing leukocytes (lymphocytes and monocytes) was obtained from healthy, de-identified, donor blood from the New York Blood Center (Long Island City, NY). Lymphocytes were isolated by separation over Ficoll-PaquePLUS (GE Healthcare) followed by washing with 10% fetal calf serum in phosphate-buffered saline (10% FCS/PBS). Cells were counted using a Guava cell analyzer (EMD Millipore), diluted to appropriate concentration and frozen at -80 °C (Revco) for 3 days, followed by long-term storage in liquid nitrogen. Upon use,
cells were thawed in 10% human serum in RPMI 1640 medium (10% HS/RPMI, Gibco) and brought up to 5x10^6 cells per ml for cell culture experiments.

2.3.2 Preparation of extracts and fractions for bioactivity testing

Extracts, fractions and pure compounds were brought to desired concentration using a mixture of DMSO and 10% HS/RPMI so that final concentration was no more than 0.5% DMSO (v/v) in each culture well.

2.3.3 Endotoxin screening

To avoid false positive results triggered by a response of PBMCs to lipopolysaccharide (LPS) contamination, samples were subjected to endotoxin screening before cell culture experiments. After extracts, fractions and isolated compounds were prepared in media they were screened using the Endosafe Limulus amebocyte lysate (LAL) automated assay (Charles River Labs) with cartridges sensitive to 0.1 EU endotoxin/ml.

2.3.4 Cell culture protocol

A half-million cells (0.5x10^6 cells in 100 uL) were added to each well of a 96-well plate. The cells were then treated with 50 µL of crude extract or fractions, prepared as in section 2.3.2. Positive control consisted of PBMCs treated with 50 µL 0.01 µg/ml *Staphylococcus* enterotoxin B (SEB) and negative control wells contained media with 0.5% DMSO. Cells were incubated for up to 72 h at 37 °C (5% CO₂).

2.3.5 Cytokine analysis

Following incubation, cells were centrifuged and cell-free supernatant was harvested. A colorimetric ELISA kit (R&D Systems) was used for initial bioactivity-guided fractionation to measure IFNγ concentration in supernatant. One modification was made to manufacturer protocol. After adding IFNγ-conjugate antibody, instead of incubating for 2 h, as recommended,
the plates were incubated for 24 h, before color reagents were added (See Appendix E for further details). The absorbance was measured at 450 and 540 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA). A multiarray electrochemiluminescence assay was used to measure all other cytokines reported in this study. The V-plex human proinflammatory cytokine plate and reagents (Meso-scale Discovery) were used with no deviation from manufacturer protocol and luminescent intensities were measured and converted to concentration of cytokine (pg/mL) using a SECTOR Imager 2400 (Meso-scale Discovery).

2.3.6 MTT protocol

To measure cell proliferation an MTT assay kit was used (Roche Applied Science, Indianapolis, IN). After incubation 10 μl of MTT labeling reagent was added to each well, and cells were incubated for another 4 h. 100 μl of solubilization solution was added to each well and the absorbance was measured at 590 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA) after overnight incubation.

2.4 LC-MS-TOF and Chemometric Analysis

2.4.1 Preparation of samples for LC-MS analysis

Extracts and fractions were brought up to 1 mg/mL using LC/MS grade methanol. After filtration through a 0.45 μM syringe filter, the fractions were further diluted with methanol to 0.25 mg/mL.

2.4.2 MS-TOF conditions

HPLC separation of crude methanol extract and fractions was performed using a Waters (Milford, MA) Alliance system with a 2695 separations module and a 2996 PDA detector. Separation was achieved on a 150 x 2.0 mm, 2.6 μm, Kinetex C-18 column (Phenomenex, Torrance, CA), held at a constant temperature of 45°C using a gradient system composed of A,
0.1% formic acid in water, and B, 0.1% formic acid in MeCN, at a flow of 0.2 mL/min: 0-5 min B, 9%; 5-10 min B, 9-20%; 10-20 min B, 20-50%; 20-30 min B, 50-90%; 30-45 min, 90-95%; 45-59 min B, 95% before returning to initial conditions and equilibrating for 10 min. Each sample was injected three times, with an injection volume of 5 ul. The results were monitored using a photodiode array (PDA) detector set to record wavelengths from 200-600 nm.

High-resolution electrospray ionization mass spectroscopy (HR-ESI-MS) was performed using a LCT Premier XE TOF mass spectrometer (Waters, Milford, MA) (LC-MS-TOF) equipped with an ESI interface, controlled by MassLynx V4.1 software. Mass spectra was acquired in positive mode over the range m/z 100–1000. The capillary voltage was set to 3000 V and the cone voltage was 20 V. Nitrogen gas was used for both the nebulizer and in desolvation. The desolvation and cone gas flow rates were 600 and 20 L/h, respectively. The desolvation temperature was 400 °C, and the source temperature was 120 °C. For the dynamic range enhancement (DRE) lockmass, a solution of leucine enkephalin (Sigma-Aldrich, St. Louis, MO) was infused by a secondary reference probe at 200 pg/mL in acetonitrile/water (1:1) containing 0.1% formic acid with a second LC pump (Waters 515 HPLC pump). The reference mass was scanned once every five scans for each positive data collection. Positive ESI data was collected using a scan time of 0.2 s, with an interscan time of 0.01 s and a polarity switch time of 0.3 s.

2.4.3 MarkerLynx

Chemometric analysis was performed using MarkerLynx XS software. Chromatographic peaks were detected in the positive mode by ApexPeakTrack, following the parameters: 8-48 min retention time, 300-800 Da mass range, mass tolerance of 40 mDa. Isotopic peaks were excluded from analysis, the noise elimination level was set at 1.00, the intensity threshold (counts) of collection parameters was set at 5000, and retention time window was set at 0.4 min.
The retention time and m/z data pair for each peak was collected and organized by ApexPeakTrack. PCA and OPLS-DA on extracted markers was conducted using the built-in MarkerLynx XS platform, with Pareto scaling.

2.5 Statistics

The Base R software package provided 'aov' and 'TukeyHSD' functions used for plotting, analysis of variance and comparison of means between sample groups (R Core Team, 2013). The 'ggplot2' package (Wickham and Chang, 2013) provided functions used to create boxplots showing cytokine response. The 'doBy' package (Hojsgaard and Halekoh, 2013) provided the function 'summaryBy' and the 'psych' package (Revelle, 2013) provided the 'describeBy' functions used to obtain group means.

3. Results

3.1 Initial bioactivity screening

To determine initial bioactivity of *Phaleria nisidai*, PBMCs were cultured with three concentrations (62.5, 125, 250 μg/mL) of crude methanol extract (PNM). After 72 h, ELISA and MTT assays were performed on these cells. PBMCs treated with *P. nisidai* extracts produced greater amounts of IFNγ (Table 2.1) than the negative control and a dose-dependent response was observed. Next, the MTT assay was used to evaluate proliferation of PBMCs. Crude extract caused cell proliferation at levels 85.4 to 134.5% higher than untreated control (Table 2.1). These experiments demonstrated the immunostimulatory activity of the crude *P. nisidai* extract and justified bioactivity-guided fractionation to identify immunoactive compounds in this extract.
Table 2.1. Effect of crude MeOH extract on PBMCs treated with varying doses of Phaleria nisidai crude extract.

<table>
<thead>
<tr>
<th>PBMC treatment</th>
<th>IFNγ (pg/mL)$^1$</th>
<th>cell proliferation (%)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>143.1 ± 37.2$^A$</td>
<td>-</td>
</tr>
<tr>
<td>crude 62.5 μg/mL</td>
<td>1228.4 ± 48.2$^B$</td>
<td>134.5 ± 2.2*</td>
</tr>
<tr>
<td>crude 125 μg/mL</td>
<td>1432.5 ± 24.4$^{BC}$</td>
<td>116.3 ± 17.6*</td>
</tr>
<tr>
<td>crude 250 μg/mL</td>
<td>1585.4 ± 0.9$^B$</td>
<td>85.4 ± 2.7*</td>
</tr>
<tr>
<td>SEB</td>
<td>647.5 ± 79.4$^E$</td>
<td>134.7 ± 13.3*</td>
</tr>
</tbody>
</table>

$^1$ Levels with different letters are significantly different ($p<0.05$)

$^2$ Percent proliferation above untreated negative control

* Indicates significant proliferation above negative control

3.2 Activity-guided fractionation

After showing activity in initial screens crude Phaleria nisidai extract was subjected to liquid-liquid partitioning and fractionation (Figure 2.3). Concentrated extract (76 g) was suspended in a mixture of 20% MeOH-H$_2$O (1.5 L) and partitioned sequentially with hexane, ethyl acetate (EtOAc), and n-butanol (BuOH), to afford hexane-, EtOAc- and BuOH- soluble extracts. The hexane-soluble portion, which had the highest activity in an initial IFNγ ELISA screen (data not shown) was prioritized for further purification (Figure 2.3).

The hexane-soluble portion (4g) was subjected to column chromatography over 160 g Sephadex® LH-20 and eluted isocratically with 100% isopropanol. Twenty-four fractions were obtained and recombined following TLC analysis to yield 6 fractions (HF1-HF6). Fractions HF5 (11.5 mg) and HF6 (13.3 mg) were most active in the ELISA assay (Figure 2.4). Due to low yields and similar chemical fingerprint by HPLC-MS-TOF these fractions were combined (HF56) and subjected to further purification. HF56 was separated over 5 g reversed-phase C18, eluting with 30% MeOH-H$_2$O to 90% MeOH-H$_2$O. Six fractions were obtained and recombined following TLC analysis. Three final fractions were obtained: 141A, eluted with 1:4 MeOH-H$_2$O; 141B, eluted with 1:1 MeOH-H$_2$O; and 141C (3.9 mg), eluted with 7:3 to 9:1 MeOH-H$_2$O. HPLC-MS-TOF revealed a similar chromatographic profile for fractions 141A and 141B, and
due to low yields they were recombined (recombined: 141AB) before screening in IFNγ ELISA and MTT cell proliferation assays. 141C was the most active fraction in the ELISA assay (Figure 2.4) with strong activity at 10 µg/ml, while 141AB was inactive (similar to negative control, $p<0.05$).

**Figure 2.3** Partition scheme, highly active fractions are in green, moderately active fractions are in yellow and inactive fractions are in white boxes.

141C and 141AB fractions were screened for cell proliferation. As shown in Figure 2.6, 141C caused a time-dependent increase in PBMC proliferation. At 48 h of treatment there was 31.9% more proliferation, and after 72 h there was 85.9% proliferation than negative control. 141AB did not cause a significant increase in proliferation at any time point.
Figure 2.4 Concentration of IFNγ in PBMC culture after treatment with *Phaleria nisidai* fractions (HF1-HF6, 141AB-C) at 10 μg/ml for 72 hours, as measured by ELISA. PBMCs incubated with 0.5% DMSO as negative control (NC) and with 0.01 μg/ml *Staphylococcus* enterotoxin B (SEB) for positive control. Results are expressed as mean ± standard error (n=3).
Figure 2.5 Concentration of IFNγ in PBMC culture after treatment with *Phaleria nisidai* fractions 141AB and 141C (10 μg/mL) and a standard acylglucosylsterol mixture at 100 μg/mL (AGS100), 10 μg/mL (AGS10) and 1 μg/mL (AGS1). PBMCs incubated with 0.5% DMSO as negative control and 0.01 μg/mL *Staphylococcus enterotoxin B* (SEB) for positive control. Results are expressed as mean ± standard error (n=3).

Figure 2.6 PBMC proliferation after treatment with fractions 141AB and 141C at 5 μg/ml after 24, 48 and 72 h. Values indicate % above negative control (MeOH treatment). *Staphylococcus enterotoxin B* (SEB) at 0.01 μg/ml used as positive control. Results are expressed as mean ± standard error (n=3).
3.3 LC-MS-TOF fingerprinting of fractions

Fractions 141A-C were obtained sequentially by separating HF56 over reversed-phase C18 media. 141A and 141B eluted first and have more peaks in the more polar region of the chromatogram. 141C, eluting last, shows peaks with a higher intensity eluting in the more nonpolar regions of the chromatogram (Figure 2.7). 861 total ions were detected in these fractions by ApexPeakTrack (MarkerLynx) using our parameters.

3.4 Chemometric identification of active compounds

An untargeted PCA analysis based on the positive mode of LC-TOF-MS data was used to generate an overview of all samples. Retention time, exact mass and ion intensity were used as variables to cluster fractions. Each fraction was injected 5 times. In two-dimensional PCA score plots (Figure 2.8) the active fractions separated into clusters distinct from inactive fractions and there was no overlap of active and inactive clusters.

Figure 2.7 LC-MS-ESI-TOF chromatograms in positive mode for fractions 141a (top), 141b (middle) and 141c (bottom).
To determine the ions that contributed highly to sample clustering OPLS-DA was used. The OPLS-DA scores plot accounted for 82.28% of the total variability (component 1, 62.72%; component 2, 19.56%). An S-plot (Figure 2.9) generated from the loadings of the OPLS-DA scores plot highlights which variables are responsible for differences between the groups. Discriminant marker candidates can be chosen from S-plots by applying a cutoff for correlation value, $p[\text{corr}]$, to $>|0.5|$ and covariance, $p[1]$, to $>|0.2|$. Marker candidates can be further confirmed by variable importance in projection (VIP) values as compounds with larger VIP values are more relevant for model construction.

**Figure 2.8** PCA scores plot for 141 fraction set. Fraction 141C as distinct cluster than 141A and 141B. Proximity of 141A and 141B indicate that they are more similar than 141C.
Figure 2.9. OPLS-DA S-plots for 141 fraction set. Markers characteristic of active fractions are highlighted, labeled and shown in inset, labels correspond to Table 2. Markers for active fraction 141C in upper right quadrant. Markers for inactive fraction 141A and B in lower left quadrant.

This analysis revealed that the most relevant compound to distinguish active fraction 141C (VIP = 5.59) from inactive 141A and 141B has exact mass \((m/z^+) = 533.3112\) (C\(_{30}\)H\(_{45}\)O\(_8\), \(t_R\): 34.8 min). This compound was only detected in the active fraction 141C and was tentatively identified as the daphnane simplexin. Other daphnanes observed with high VIP values are present only in 141C (labeled markers in Figure 2.9) and not in inactive fractions.

3.5 Identification of marker compounds

The marker eluting at \(t_R\): 34.8 min with \(m/z^+ = 533.3112\) was tentatively identified as simplexin (see Table 2.2). A pure standard of simplexin, obtained from Dr. Mary Fletcher (Queensland Primary Industries and Fisheries, Animal Research Institute, Australia) was used to confirm identity by coinjection experiments.

Other compounds contributing to the chemical difference of the active fraction were tentatively identified as related diterpene orthoesters (Table 2.2) based on exact mass of
pseudomolecular ions, adducts and losses on LC-MS-TOF and tandem MS on UPLC-MS-TQD (See Chapter 3). These compounds have been previously reported to exist in other plants from the same subfamily, Thymelaeoideae (Borris et al., 1988), within Thymelaeaceae. Structures of all marker compounds can be found in Figure 2.10.

3.6 Cytokine modulation by simplexin and Phaleria nisidai treatments

To test the hypothesis that simplexin contributes highly to the immunostimulant activity observed in Phaleria nisidai the pure compound was used as a treatment in PBMC cell culture and output of multiple cytokines was measured using a multiarray electrochemiluminescence cytokine assay. The levels of eight cytokines were measured and are reported in Table 2.3. PBMCs were cultured with the treatment alone or treatment + CEF peptides, a pool of epitopes used to generate specific immune response.

The fraction 141C increased the production of the IFNγ, IL1B, IL2, IL4, IL6, IL10 and IL13 above negative control. Simplexin increased the production of IFNγ, IL6 and IL13 above negative control. Prostratin was used as a reference compound and caused more IL4 and IL6 production than simplexin, while simplexin caused more IFNγ production than prostratin. Cotreatment with CEF peptides did not cause any significant change in cytokine production for fraction 141C, simplexin, prostratin or negative control.

Plots showing the concentration for all eight cytokines as a result of crude extract, 141C and simplexin treatment are shown in Figure 2.12. The same pattern is observed for all cytokines; as purity of the treatment increases, and simplexin becomes more enriched, activity increases. Simplexin at 1 μg/mL has equivalent activity to 141C at 10 μg/mL for all cytokines, with the exception of IL10. 141C causes more IL10 production than simplexin in PBMC culture.
Figure 2.10 Structures of 6 daphnane orthoesters identified from active fraction 141C.
Table 2.2. Tentative identification of marker compounds for active fraction 141C.

<table>
<thead>
<tr>
<th>Marker (from Fig 4)</th>
<th>R.T. (min)</th>
<th>Parent ion, ppm</th>
<th>Adduct and fragmental ion exact masses [M-X]+ or [M-X]- (molecular formula, ppm)</th>
<th>Tentative identification [metabolite class]</th>
<th>Notes</th>
<th>VIP value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34.40</td>
<td>533.3115[M+H]+ ((C_{30}H_{45}O_8, 0.2))</td>
<td>531.3023[M-H]- ((C_{30}H_{43}O_8, 12.2))</td>
<td>simplexin (co-injection) [daphnane]</td>
<td>previously reported from <em>Daphne</em> and <em>Pimelea</em> genera (Hecker, 1977)</td>
<td>5.59</td>
</tr>
<tr>
<td>F</td>
<td>34.63</td>
<td>625.2649[M+H]+ ((C_{31}H_{41}O_{11}, 0.6))</td>
<td>623.2492[M-H]- ((C_{31}H_{40}O_{11}, 0.6))</td>
<td>unidentified</td>
<td>previously reported from <em>Daphne genkwa</em> (Zhan et al., 2005); less polar, eluting second of two genkwanine isomers (Geng et al., 2013)</td>
<td>4.01</td>
</tr>
<tr>
<td>C</td>
<td>34.32</td>
<td>609.2707[M+H]+ ((C_{34}H_{41}O_{10}, 1.1))</td>
<td>607.2531[M-H]- ((C_{34}H_{39}O_{10}, 2.0))</td>
<td>genkwanine H [daphnane]</td>
<td>previously reported from <em>Daphne genkwa</em> (Zhan et al., 2005); more polar, eluting first of two genkwanine isomers (Geng et al., 2013)</td>
<td>4.93</td>
</tr>
<tr>
<td>E</td>
<td>34.95</td>
<td>609.2715[M+H]+ ((C_{34}H_{41}O_{10}, 2.5))</td>
<td>607.2532[M-H]- ((C_{34}H_{39}O_{10}, 1.8))</td>
<td>genkwanine D [daphnane]</td>
<td>previously reported from <em>Daphne genkwa</em> (Zhan et al., 2005); less polar, eluting second of two genkwanine isomers (Geng et al., 2013)</td>
<td>4.25</td>
</tr>
<tr>
<td>B</td>
<td>35.49</td>
<td>593.2763[M+H]+ ((C_{36}H_{51}O_{10}, 0.5))</td>
<td>591.2559[M-H]- ((C_{36}H_{49}O_{10}, 2.4))</td>
<td>unidentified</td>
<td>previously reported from <em>Pimelea</em> spp. (Freeman et al., 1979), <em>Synaptolepis</em> spp. (Adolf et al., 1988) and <em>Wikstroemia retusa</em></td>
<td>5.16</td>
</tr>
<tr>
<td>D</td>
<td>35.96</td>
<td>643.3477[M+H]+ ((C_{36}H_{49}O_{10}, 0.8))</td>
<td>643.2310[M-H]- ((C_{36}H_{48}O_{10}Cl, 1.1))</td>
<td>acetoxylhuratoxin [daphnane]</td>
<td></td>
<td>4.75</td>
</tr>
<tr>
<td>G</td>
<td>34.91</td>
<td>601.3385[M+H]+ ((C_{34}H_{49}O_{10}, 1.3))</td>
<td>599.3220[M-H]- ((C_{34}H_{47}O_{10}, 8.3))</td>
<td>stelleramacrin B [daphnane]</td>
<td>previously reported from <em>Stellera chamaejasme</em> (Ikekawa and Ikekawa, 1994; Weijian et al., 1997)</td>
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<tr>
<td>H</td>
<td>36.99</td>
<td>585.3430[M+H]+ ((C_{34}H_{49}O_{10}, 0.5))</td>
<td></td>
<td>huratoxin [daphnane]</td>
<td>previously reported from <em>Pimelea simplexin</em> (Freeman et al., 1979), <em>Stellera chamaejasme</em> (Tatematsu et al., 1984) and <em>Wikstroemia monotica</em> (Jolad et al., 1983)</td>
<td>3.15</td>
</tr>
</tbody>
</table>
Table 2.3. Concentration of cytokines (pg/mL) in response to simplexin and prostratin. SEB used as positive control. NC = negative control (DMSO and media treatment only). Results reported as ± SE.*indicates simplexin and prostratin treatments are different (p<0.05). N indicates treatment is greater than NC.

<table>
<thead>
<tr>
<th>cytokine</th>
<th>simplexin alone</th>
<th>prostratin alone</th>
<th>SEB alone</th>
<th>NC alone</th>
<th>SEB with CEF</th>
<th>NC with CEF</th>
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</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>4433.1 ± 1306.2*</td>
<td>3650.2 ± 833.5</td>
<td>1433.7 ± 269.5*</td>
<td>2177.5 ± 374.0</td>
<td>13022 ± 4541</td>
<td>12432 ± 5867</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32.7 ± 23.0</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>872.0 ± 547.7</td>
<td></td>
</tr>
<tr>
<td>IL10</td>
<td>2.6 ± 1.2</td>
<td>4.7 ± 0.9</td>
<td>4.7 ± 1.2*</td>
<td>3.3 ± 0.6</td>
<td>12.7 ± 2.6</td>
<td>15.1 ± 7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>IL12</td>
<td>2.1 ± 0.8</td>
<td>3.8 ± 0.9</td>
<td>2.5 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>2.7 ± 0.4</td>
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<td></td>
<td></td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.5</td>
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<tr>
<td>IL13</td>
<td>389.3 ± 122.0*</td>
<td>365.9 ± 78.7</td>
<td>231.7 ± 74.7</td>
<td>196.2 ± 62.2</td>
<td>64.3 ± 5.2</td>
<td>66.2 ± 9.2</td>
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<td></td>
<td>15.0 ± 1.8</td>
<td>14.8 ± 3.0</td>
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<tr>
<td>IL1b</td>
<td>477.7 ± 147.2</td>
<td>447.8 ± 97.0</td>
<td>795.7 ± 200.3*</td>
<td>784.3 ± 192.7</td>
<td>116.5 ± 75.1</td>
<td>27.7 ± 9.7</td>
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<td></td>
<td></td>
<td></td>
<td>9.8 ± 3.3</td>
<td>19.7 ± 9.4</td>
</tr>
<tr>
<td>IL2</td>
<td>6.3 ± 1.8</td>
<td>7.9 ± 1.4</td>
<td>6.5 ± 1.9</td>
<td>4.9 ± 0.4</td>
<td>50.5 ± 12.2</td>
<td>26.9 ± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.2 ± 0.9</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>IL4</td>
<td>0.3 ± 0.2*</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.2*</td>
<td>1.9 ± 0.7</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>IL6</td>
<td>1070.8 ± 253.3*</td>
<td>1241.1 ± 152.6</td>
<td>1970.4 ± 121.1*</td>
<td>1983.4 ± 125.2</td>
<td>1013.2 ± 385.0</td>
<td>528.4 ± 224.8</td>
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<td></td>
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<td></td>
<td></td>
<td>86.9 ± 34.5</td>
<td>216.8 ± 110.6</td>
</tr>
</tbody>
</table>

**Figure 2.11** Concentration (pg/mL) of T_{H1} cytokines in PBMC supernatant following treatment with *P. nisidai* crude extract (200 µg/mL), 141C fraction (10 µg/mL), simplexin (1 µg/mL), prostratin (1 µg/mL) and SEB (0.01 µg/mL). Media is used as negative control (NC). White dot indicates group mean.
Figure 2.12 Concentration (pg/mL) of $T_h2$ cytokines in PBMC supernatant following treatment with *P. nisidai* crude extract (200 μg/mL), 141C fraction (10 μg/mL), simplexin (1 μg/mL), prostratin (1 μg/mL) and SEB (0.01 μg/mL). Media is used as negative control (NC). White dot indicates group mean.

4. Discussion

A simple *in vitro* ELISA assay to measure IFNγ was used as a preliminary screen to classify fractions in a binary manner as active or inactive. Principal component analysis was used to cluster fractions based on chemical profile. OPLS-DA was used to generate an S-plot, highlighting individual components making high contributions to differences in the chemical composition of fractions in these clusters. Using these strategies, simplexin was predicted to be an active compound in 141C, because it had the largest VIP (variable importance in projection) value.
A similar approach has been used by other groups to identify adenosine A1 receptor-binding compounds from *Boesenbergia rotunda* extracts with different polarity (Yuliana et al., 2013), thrombin-induced phosphorylation inhibitors from 43 green tea cultivars (Fujimura et al., 2011) and TNFα-inhibitory compounds from grapes and other berries (Ali et al., 2012). These chemometric techniques allow for the identification of putative active compounds while avoiding the time, energy and resource-intensive methods of bioactivity-guided isolation, in which it is necessary to purify many compounds using large volumes of solvent, followed by assays using each isolated compound, to determine the compound responsible for activity in a natural product.

After cytokine analysis of PBMC culture supernatant, it was observed that simplexin caused an increase in IFNγ at low dose (1 μg/mL), indicating that this chemometric approach to active compound identification was successful. IFNγ was monitored because it is the key cytokine for generation of a T\textsubscript{H}1 response (Mosmann et al., 1986), representative of cell-mediated immunity and macrophage activation (Romagnani, 1999). This response contributes to direct antimicrobial and antitumor immunity in a system (Schroder et al., 2004). The effects of simplexin on seven other cytokines were also determined using a multiarray cytokine assay as well to obtain a broader picture of its effects on PBMCs. The biological effects of these cytokines are summarized in Table 2.4 and Figure 2.13.

IL6 and IL13 were increased as a result of simplexin treatment. By contrast, these cytokines are indicative of a T\textsubscript{H}2 response, representative of humoral, antibody-dependent immunity (Romagnani, 1999). PBMCs represent a subset of white blood cells containing natural killer cells, monocytes and macrophages, and T- and B-lymphocytes. It is unclear exactly which cells are stimulated by simplexin treatment and responsible for the observed effects, however it
is clear that simplexin, as well as the 141C fraction, have activity in stimulating both cell- and antibody-mediated immunity.

**Figure 2.13** T\textsubscript{H}1/T\textsubscript{H}2 cytokines, their activity and interplay (Murphy et al., 2007; Romagnani, 1999, 1996)

Fraction 141C increased IL4 and IL10, two other T\textsubscript{H}2 cytokines. The many components in this fraction help contribute to a different cytokine profile than pure simplexin alone. Some components of 141C may exert anti-inflammatory activity, while others may contribute to a more T\textsubscript{H}1 cell-mediated, inflammatory response, which can explain the observed increase in IL1B and IL2 seen with this treatment. These dichotomous results suggest that the active ingredients in *Phaleria nisidai* can help the immune system to protect against pathogens and
tumor, but also to prevent undesired inflammation and autoimmune reactions, depending on the specific cells activated and immune status at the time of consumption.

Table 2.4 Biological effects of cytokines monitored in this study (Murphy et al., 2007)

<table>
<thead>
<tr>
<th>cytokine</th>
<th>produced by</th>
<th>effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>lymphocytes</td>
<td>macrophage activator</td>
</tr>
<tr>
<td>IL-1β</td>
<td>activated macrophages</td>
<td>stimulates thymocyte production, IL-2 release, B-cell maturation and proliferation, fibroblast growth factor</td>
</tr>
<tr>
<td>IL-2</td>
<td>T-cells</td>
<td>&quot;T-cell growth factor&quot;</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells</td>
<td>activates B-cells, induce Th2 differentiation</td>
</tr>
<tr>
<td>IL-6</td>
<td>T-cells (released during muscle cells in exercise)</td>
<td>differentiate B-cells into Ig-secreting cells</td>
</tr>
<tr>
<td>IL-10</td>
<td>T-cells, macrophages, mast cells</td>
<td>suppressant of macrophage function, inhibit synthesis of Th1-suppressing cytokines</td>
</tr>
<tr>
<td>IL-12</td>
<td>macrophages and T lymphocytes</td>
<td>activates T cells and NK cells that stimulate IFNγ production, induces CD4 differentiation into Th1-like cells</td>
</tr>
<tr>
<td>IL-13</td>
<td>T cells and others</td>
<td>B cell growth and differentiation, inhibitor of inflammatory cytokine production by macrophages and Th1 cells, macrophage activation</td>
</tr>
</tbody>
</table>

Prostratin was chosen as a positive control in these cytokine assays because it is structurally related to simplexin, although in the tigliane, not daphnane, class of diterpene esters. Bioactivity-guided fractionation of both organic stem extracts and healer infusions of Homalanthus nutans, a tree in the family Euphorbiaceae used to treat hepatitis and yellow fever in Samoa resulted in the isolation of prostratin (Gustafson et al., 1992; Johnson et al., 2008). This compound was found to have potent activity against HIV-1 infection in T cell-based assays conducted by the NCI (National Cancer Institute, Frederick, MD). Prostratin has promise in eradicating latent HIV viral reservoirs from T-cells and is in pre-clinical screening.

Simplexin caused greater IFNγ release than prostratin, while prostratin caused greater IL1β release by PBMCs. These are both TH1 cytokines, but are released by different cells (Table...
As for T\textsubscript{H}2 cytokines, prostratin caused more IL4, IL6 and IL10 production than simplexin, while simplexin caused more IL13 production. It appears that prostratin and simplexin have distinct cytokine profiles; which include upregulation of certain T\textsubscript{H}1 or T\textsubscript{H}2 cytokines. The complex results observed in this study indicate that although both of these diterpenes cause a unique pattern of cytokine release, they can both contribute to cell-mediated or humoral immunity; with both inflammatory and antiinflammatory effects. Further experiments need to be performed to determine the effects of these compounds in more controlled systems, such as on one cell type as can be performed by flow cytometry.

Simplexin and five other daphnanes are reported here for the first time from Phaleria nisidai Kaneh., and is the first report of daphnanes from this genus. Tigliane, ingenane and daphnane diterpenes are commonly found in, but restricted to, the Thymelaeaceae and Euphorbiaceae families with daphnanes mostly found in plants from the Thymelaeaceae rather than the Euphorbiaceae family (Borris et al., 1988; He et al., 2002). According to a recent review 100 daphnanes have been isolated from 10 genera of the Thymelaeaceae and Euphorbiaceae families (Wang et al., 2013). Other Thymelaeaceae genera that produce daphnanes include Pimelea, Gnidia, Daphne, Daphnopsis, Synaptolepis, Diarthron, Peddiea, Stellera, and Wikstroemia, which are all in the subfamily Thymelaeoideae, along with Phaleria according to the taxonomical treatment by Gilg (Borris et al., 1988). These results further confirm that that Thymelaeoideae subfamily contains daphnane-producing plants.

Many daphnanes have shown anti-HIV activity (Asada et al., 2011; Cheng et al., 2013; Huang et al., 2011, 2012; Vidal et al., 2012) and simplexin has shown inhibition of HIV replication combined with low cytotoxicity in an MT4 T-cell line (Asada et al., 2011). Mechanism of anti-HIV action for the tigliane prostratin and the daphnane gnidimacrin includes
blocking HIV entry into cells (Xing and Siliciano, 2013) through down-regulation of chemokine cell surface receptors CXCR4 and CCR5, which are also co-receptors for HIV (Vidal et al., 2012). This occurs through the typical protein kinase C (PKC) activation observed with many diterpene esters (Hecker, 1985; Huang et al., 2011; Liao et al., 2009). It is characteristic of PKC activators to cause release of cytokines in PBMCs (Xing and Siliciano, 2013) and simplexin has been shown to promote maturation of human promyelocytic cells (HL-60) into more mature monocytes (Nakayasu et al., 1982). It is evident that daphnanes exert activity on T cells and PBMCs and results of this work are in agreement with bioactivity reported in the literature, most likely through activation of the PKC pathway.

Simplexin was initially isolated in 1977 from Pimelea simplex, P. elongata (Zayed et al., 1977), and P. trichostachya (Wilson et al., 2007), three species responsible for the livestock poisoning in Australian cattle known as St. George Disease (Fletcher et al., 2009; Freeman et al., 1979; Seawright, 1984). Activity-guided isolation found simplexin and other daphnanes to be responsible for this disease (Freeman et al., 1979; Roberts et al., 1975). While it is essential to mention cases of animal and in vitro toxicity, this compound has never been shown to have toxicity towards humans in clinical or case studies. A larger discussion of the toxicity of simplexin can be found in Chapter 3.

Many tigliane and daphnane diterpene esters have tumor-promoting activity. They are not carcinogenic themselves but when administered following exposure to a subeffective dose of a carcinogen (usually the initiator DMBA), lead to the appearance of skin tumors, shown mostly in mice (Blumberg, 1988). A clear structure-activity relationship exists in which esters with different aliphatic moieties possess striking differences in tumor-promotion assays (Blumberg,
Prostratin, a reference compound in this study, is one diterpene ester that is not tumor-promoting (Gustafson et al., 1992).

Simplexin has been shown to be both nonirritant (Zayed et al., 1977) and irritant (Adolf et al., 1988) in mouse ear models, and a moderate cocarcinogen in mouse skin (Zayed et al., 1982). At the same time, this compound has also demonstrated potent inhibition of the murine P-388 lymphocytic leukemia cell line (Pettit et al., 1983). Possibly correlating with our observation of PBMC proliferation and IFNγ production, simplexin has caused differentiation of human promyelocytic cells (HL-60) to more mature monocytes in vitro (Nakayasu et al., 1982). These superficial differences observed in bioactivity highlight that important immune and inflammatory mechanisms may be targeted by this compound and it should not be disregarded as another toxic diterpene ester without beneficial pharmaceutical qualities, just as prostratin was once in danger of being excluded from further studies (Cox, 2001) because it was a phorbol tigliane diterpene ester.

Questions concerning the pharmacology and toxicology of simplexin remain, and can be answered with further investigations of its toxicity, especially towards humans, and its mechanism of action and effects on specific immune cells. From an ethnomedicinal and public health perspective further work needs to be performed to determine if simplexin and other potentially toxic daphnane orthoesters are present in appreciable levels in water extracts, the primary traditional preparation of this plant. Chapter 3 quantifies the levels of simplexin and two other daphnanes in water extracts of this plant.

Following bioactivity-guided fractionation and isolation Matsuda's group determined that a mixture of acylglucosylsterols, with different fatty acids esterified to the core sterol, (Chapter 1, Figure 1.7) were responsible for immunostimulatory activity of a 50% ethanol extract of
Phaleria nisidai (Chapter 1, Figure 1.8). The acylglucosylsterol found by Matsuda's group (2005b) was reported to have an exact mass = 837.6563 (calculated for [M+Na]^+ = C_{51}H_{90}O_{7}Na) by fast-atom bombardment mass spectrometry (LC-FAB-MS). Neither this mass, nor its protonated ion ([M+H]^+=C_{51}H_{91}O_{7}), were detected in HF or 141 fractions of *P. nisidai* using our electrospray ionization mass spectrometry (LC-ESI-MS) technique. We employ a different ionization technique than Matsuda, which may explain why we cannot detect these compounds in the eight fractions analyzed in this study. Another explanation is that these compounds are not present in the hexane partition that these fractions were derived from.

To put these findings in the context of Matsuda's work on *Phaleria nisidai*, a standard mixture of acylglucosylsterols (Matreya LLC), with different fatty acids esterified to the core sterol, was tested for activity in the IFNγ ELISA assay following culture with PBMCs. This standard mixture was active, producing more IFNγ than PBMCs treated with negative control, but caused significantly less IFNγ production than fraction 141C (10 μg/mL) (Figure 2.5). The acylglucosylersterol mixture at its most active dose (AGS1; 1 μg/mL) caused 333.2 pg/mL IFNγ to be produced, compared to 1796.0 pg/mL IFNγ produced in PBMCs treated with 141C. Simplexin (1 μg/mL) had similar activity to 141C (10 μg/mL) in the electrochemiluminescence assay (Figures 2.11 and 2.12). Based on this result, we would expect simplexin to have greater activity than acylglucosysterols if compared in the same experiment.

To conclude, simplexin is an active immunomodulatory compound found in the crude methanolic extract and nonpolar fractions of *Phaleria nisidai*. These fractions have greater immunostimulatory activity than acylglucosysterols previously reported as being immunostimulatory in *Phaleria nisidai*. Simplexin and other daphnanes were reported from this genus for the first time, and they may be the compounds responsible for traditional use of *P.*
*nisidai* although further work to determine the amounts of these compounds in traditional aqueous preparations needs to be performed, and is carried out in the next chapter.
Chapter 3

Traditional preparations of *Phaleria nisidai*, an adaptogenic beverage from Palau, minimizes content of potentially toxic daphnane diterpene esters

1. Introduction

*Phaleria nisidai* Kaneh. (Thymelaeaceae) is one of the most popular and well-respected medicines in Palau (Dahmer et al., 2012). It is known locally as *delalakar*, which translates to "the Mother of medicine." Leaves of *P. nisidai* are picked and boiled fresh to make a medicinal tea used as a strengthening remedy and energizing tonic (Dahmer et al., 2012) as well as to keep away sickness and boost the immune system (see Chapter 1). Many people consume it every day and some drink this beverage in lieu of water. Our primary ethnobotanical research indicates that most people report this preparation as possessing no dangerous side-effects despite almost chronic administration for some users of this medicine (Chapter 1, Table 1.3).

One group has previously engaged in pharmacological studies of *Phaleria nisidai* and they have demonstrated immunostimulatory activity in 50% ethanolic leaf extracts of *P. nisidai* in *vivo* and in *vivo* (Matsuda et al., 2005a, 2004). Specifically the plant extract was shown to increase macrophage activity and the production of pro-inflammatory, immune-stimulating cytokines. In these studies *P. nisidai* ethanolic extracts and fractions were shown to enhance immune function, decrease tumor size and inhibit cell proliferation; and a mixture of acylglucosylsterols was determined to be responsible for activity (Matsuda et al., 2005a). There was no reported toxicity in any of the animals used in the study.

Previously, the production of IFNγ by PBMCs was used to guide fractionation of a methanol extract of *Phaleria nisidai*. The multivariate analytical approach of orthogonal partial
least squares-discriminatory analysis (OPLS-DA) of active vs. inactive fractions determined that simplexin and other daphnanes were present in *P. nisidai* and were important marker ions distinguishing active from inactive fractions.

Simplexin is a daphnane, a type of diterpene. Members of this class present interesting bioactivities, especially against cancer (Liao et al., 2009) however an obstacle towards their further pharmaceutical development is that many daphnane diterpene esters have irritant and tumor-promoting activity. Simplexin has shown moderate activity as an irritant (mouse ear model) and a co-carcinogen (Adolf and Hecker, 1982; Hafez et al., 1983); however has shown only very low levels of tumor promotion in the absence of the tumor initiator DMBA (Hafez et al., 1983). In fact, most daphnanes do not show any tumorigenic activity on their own (Liao et al., 2009). While simplexin's role as carcinogen is equivocal, it is perhaps most well-known as the causative agent of St. George disease. This disease occurs in grazing animals upon ingestion of several plants from the *Pimelea* genus (Thymelaeaceae), that contain simplexin. This sometimes fatal disease results in chronic weight-loss, edema and diarrhea evident of gastrointestinal distress, which can be reversed by moving the animals to new grazing territory free of *Pimelea* plants. If the disorder is not mitigated quickly enough death occurs due to pulmonary vein hypertension (Fletcher et al., 2009).

A nine mg injection of simplexin into a 100 kg calf caused death within 0.5 h and in mice the compound had an LD$_{50}$ of 1 mg/kg (Freeman et al., 1979). St. George disease has also been reported in horses, and the insult is believed due to severe acute gastrointestinal irritation (Wilson et al., 2007). Despite the reports of gastrointestinal and pulmonary toxicity attributed to simplexin in other large mammals, no human toxicity has been reported from this compound to the best of our knowledge. Most reports from research participants during ethnobotanical
interviews indicate that the medicinal beverage made from *Phaleria nisidai* leaves does not show any human toxicity, however two participants mentioned diarrhea as a side effect with one mentioning death if too much is consumed (Chapter 1, Table 1.1). To better understand the public health implications of this important, widely-used medicinal beverage this study compares the bioactivity and chemistry of methanol and traditional water extracts of *P. nisidai*, a plant used habitually by many in Palau.

![Compound Structures](image)

<table>
<thead>
<tr>
<th>compound</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>simplexin</td>
<td>R₁: C₉H₁₇</td>
</tr>
<tr>
<td></td>
<td>R₂: H</td>
</tr>
<tr>
<td>huratoxin</td>
<td>R₁: (CH=CH)₂(CH₂)₈CH₃-(E,E)</td>
</tr>
<tr>
<td></td>
<td>R₂: H</td>
</tr>
<tr>
<td>acetoxyhuratoxin</td>
<td>R₁: (CH=CH)₂(CH₂)₈CH₃-(E,E)</td>
</tr>
<tr>
<td></td>
<td>R₂: AcO</td>
</tr>
</tbody>
</table>

**Figure 3.1.** Structures of daphnanes quantified in crude *Phaleria nisidai* extracts

2. Materials & Methods

2.1 Plant material

Samples of *Phaleria nisidai* were collected throughout Palau from March to July 2012. Leaves from 229 trees were sampled and georeferenced. Local expert and botanist Ann Hillman-Kitalong (Belau National Museum) performed sample identification and verification. Voucher specimens were prepared and deposited at Belau National Museum, Natural History Department (Collections DK001-DK034). Palauan intellectual property rights are reserved according to material transfer agreement docket # TCO-09B5012-MTA between the City University of New
York and the Republic of Palau. Leaves were placed in an oven heated by four 30W incandescent light bulb until dry and stored at room temperature until shipment and extraction.

2.2 Extraction

One dry leaf from each of twenty seven *Phaleria nisidai* trees, randomly selected, from the above collection were combined and ground to powder in a spice mill. Six 50 mg aliquots of the ground sample were weighed out and transferred to six centrifuge tubes. Reagent grade MeOH (25 mL) was added to three of these samples, which were extracted under ultrasonic conditions for 30 minutes. Twenty-five mL HPLC-grade water was added to the remaining three samples, which were boiled in a sandbath at 100 °C for 30 mins. All samples were then centrifuged for 7 minutes at 3000 rpm. 5 mL of the extracts were decanted into vials, leaving the pellet behind. MeOH extracts were dried under N₂ and water extracts were frozen at -80 °C and lyophilized until dry.

Traditional extracts were prepared by five local healers in Palau. Seven fresh leaves were placed into one gallon of water and boiled on the stove for 20-30 min. Individual infusions were decanted into plastic water bottles and frozen for shipment to the phytochemistry lab in polystyrene coolers on dry ice. When the samples arrived at the lab aliquots were transferred to vials, frozen at -80 °C and lyophilized until dry.

Dried methanolic extracts were resuspended in MS-grade MeOH to 5 mg/mL, and passed through a .45 μm filter. Dried laboratory water extracts and traditional water infusions were resuspended in MS-grade H₂O to 5 mg/mL and passed through a .45 μm filter.

2.3 UPLC-MS-TQD equipment and conditions

Samples were analyzed by LC-MS-TQD methods using a Waters Acquity UPLC (Waters Corp., Milford, MA) module. LC separations was conducted using a Waters Acquity UPLC
Sample Manager coupled to a Waters Acquity Binary Solvent Manager. Separation was achieved on a 50 x 2.1 mm, 1.7 μm, Kinetex C18 (Phenomenex, Torrance, CA) column, held at a constant temperature of 40 °C. The mobile phase was composed of A, 0.1% formic acid in water and B, MeCN, at a flow of 0.5 mL/min with a gradient as follows: \( t_0, 50\%\ A; t_3, 95\%\ A; t_6, 95\%\ A; t_7, 50\%\ A; t_{10}, 50\%\ A.\)

MS/MS detection was made using a Waters Acquity TQD tandem triple quadrupole mass spectrometer. Ionization was achieved using a multimode source in positive electrospray (ESI) mode at the following conditions: capillary voltage at 3.0 kV, cone voltage at 30 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 50 L/h, and desolvation gas flow of 500 L/h. The desolvation and cone temperatures were set at 450 and 150 °C, respectively. Argon was used as MS/MS collision gas with a flow rate of 0.15 mL/min and collision energy set to 20 eV.

2.4 Calibration curves

Simplexin, isolated by colleagues from *Pimelea simplex*, was shipped to our lab. A stock solution was prepared and each concentration used in the standard curve was prepared by direct dilution from the stock standard. Each concentration was injected four times and the calibration curve was constructed from the mean of each concentration. A low concentration and high concentration calibration curve was prepared. Acetoxyhuratoxin and huratoxin were quantified according to the simplexin calibration curve. Simplexin and huratoxin were quantified using the lower calibration curve (five points, 108.4 to 650.4 ng/mL). Acetoxyhuratoxin was quantified using the upper calibration curve (five points, 650 and 3613.3 ng/mL).
2.5 Sample preparation for bioactivity testing

Methanol and lab-prepared water extracts were prepared for cell culture experiments. Samples were resuspended in DMSO, and then diluted to proper concentration with 10% HS/RPMI media so the final concentration of DMSO in each well was no more than 0.5% v/v.

2.6 Endotoxin screening

After botanical treatments were prepared they were screened using the Endosafe Limulus amebocyte lysate (LAL) automated assay (Charles River Labs) with cartridges sensitive to 0.1 EU endotoxin/ml.

2.7 Preparation of cells for bioactivity testing

Buffy coat containing leukocytes was obtained from healthy de-identified donors from the New York Blood Center (Long Island City, NY). Lymphocytes were isolated by separation over Ficoll-PaquePLUS (GE Healthcare) followed by washing with 10% fetal calf serum in phosphate-buffered saline (10% FCS/PBS). Cells were counted using a Guava cell analyzer (EMD Millipore), diluted to appropriate concentration with freezing buffer and frozen in -80 °C (Revco) for 3 days, followed by long-term storage in liquid nitrogen. Upon use, cells were thawed in 10% human serum in RPMI 1640 medium (Gibco) and brought up to 5x10^6 cells per ml concentration for cell culture.

2.8 Cell culture protocol

0.5x10^6 cells (in 100 uL) were added to each well of a 96-well plate. The cells were then treated with 50 uL Phaleria nisidai methanol or water extracts for a final concentration of 62.5, 125 and 250 μg/mL. Positive control consisted of PBMCs treated with 0.01 μg/ml Staphylococcus enterotoxin B (SEB) and negative control wells contained media with 0.5%
DMSO. All treatments were evaluated in triplicate culture experiments. Cells were incubated for up 72 h at 37 °C (5% \( \text{CO}_2 \)).

2.8 IFN\( \gamma \) evaluation

After incubation cells were centrifuged and supernatant was collected. A colorimetric ELISA kit (R&D Systems) was used to measure IFN\( \gamma \) concentration in supernatant. One modification was made to manufacturer protocol. After adding IFN\( \gamma \)-conjugate antibody, instead of incubating for 2 h, as recommended, the plates were incubated for 24 h, before color reagents were added. The absorbance was measured at 450 and 540 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.9 MTT protocol

To measure cell proliferation an MTT assay kit was used (Roche Applied Science, Indianapolis, IN). After incubation 10 \( \mu \)l of MTT labeling reagent was added to each well, and cells were incubated for another 4 h. 100 \( \mu \)l of solubilization solution was added to each well and the absorbance was measured at 590 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA) after overnight incubation.

2.10 Statistical analysis and graphing.

Microsoft Excel was used to plot ELISA and MTT data. The Base R software package provided 'aov' and 'TukeyHSD' functions used for plotting, analysis of variance, and comparison of means between sample groups (R Core Team, 2013). The 'doBy' package (Hojsgaard and Halekoh, 2013) provided the function 'summaryBy' and the 'psych' package (Revelle, 2013) provided the 'describeBy' functions used to obtain group means.
3. Results

3.1 Cytokine response

Organic and aqueous extracts were added to PBMCs in culture and their production of IFNγ was measured using colorimetric ELISA techniques and compared to each other and with negative control (Figure 3.2).

At doses of 62.5, 125 and 250 μg/ml the methanol extract exhibited strong bioactivity, significantly greater than untreated cells. The results hinted at dose response, with 250 μg/ml dose causing significantly more cytokine production (1585.4 pg/ml) than the 62.5 μg/ml dose (1228.4 pg/ml). The 125 μg/ml had intermediate response levels (1432.5 pg/ml) to the higher and lower doses, but was not significantly different than either. All methanol extract doses showed higher activity than aqueous extracts.

![Graph showing IFNγ production](image)

**Figure 3.2** Concentration of IFNγ (pg/mL) in PBMC culture as a result of treatment with *P. nisidai* methanol and water extracts. Negative control (NC) was treatment with DMSO and media. SEB was used as positive control at a dose of 0.01 μg/ml. Results expressed as mean ± standard error.
Although the aqueous extract caused lower cytokine production than methanol extract it still showed significantly higher activity than negative control. At 62.5 and 125 µg/ml dose a two-fold increase IFNγ production was observed (317.5 and 308.5 pg/ml respectively) than untreated cells (143.1 pg/ml) while the 250 µg/ml dose showed greater than three-fold increase (502.7 pg/ml).

3.2 Cell proliferation

Water and methanol extracts both increased cell proliferation above negative control. Figure 3.3 presents results from the MTT cell proliferation assay as % proliferation increase above untreated cells. At a dose of 62.5 µg/ml methanol extract (134.5%) was more proliferative than water (88.2%) towards PBMCs, but at 125 and 250 µg/ml both extracts had similar proliferative activity. For the methanol extract there appears to be a decrease in activity as the dose increases but the decrease is not significant.

![Figure 3.3 PBMC proliferation after treatment with crude methanol and crude water (PNW) extract at three doses. Values indicate % above negative control (MeOH treatment). Staphylococcus enterotoxin B (SEB) at 0.01 µg/ml used as positive control. Results expressed as mean ± standard error (n=3).](image)

65
3.3 Quantitation by UPLC-MS-TQD

3.3.1 Daphnanes in MeOH

Simplexin was positively identified in methanol extracts based on MS and MS/MS transitions and retention time (Table 3.1) comparison to standard compound. In triple quadrupole mass spectrometric analysis simplexin eluted at a retention time of 2.36 min. Relative retention times and the transitions seen in Table 3.1 were used to identify two additional daphnanes; huratoxin and acetoxyhuratoxin in methanol extracts. These daphnanes were quantified using a semi-quantitative approach, using the calibration curve generated from simplexin standard.

Yields of daphnanes are listed in Table 3.2.

Table 3.1 TQD-MS parameters used for quantification of daphnane diterpene orthoesters

<table>
<thead>
<tr>
<th>compound</th>
<th>RT (min)</th>
<th>quantification SRM</th>
<th>confirmation SRM</th>
<th>LOD ng/mL</th>
<th>LOQ ng/mL</th>
<th>slope</th>
<th>intercept</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>simplexin</td>
<td>2.36</td>
<td>533.3 &gt; 253.3</td>
<td>533.3 &gt; 267.2</td>
<td>30.4</td>
<td>90</td>
<td>.3337</td>
<td>4.340</td>
<td>.999</td>
</tr>
<tr>
<td>huratoxin$^a$</td>
<td>2.87</td>
<td>585.5 &gt; 253.3</td>
<td>585.5 &gt; 267.2</td>
<td>.3337</td>
<td>4.340</td>
<td>.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetoxyhuratoxin$^b$</td>
<td>2.75</td>
<td>643.3 &gt; 207.2</td>
<td>643.3 &gt; 277.2</td>
<td>.2505</td>
<td>81.48</td>
<td>.999</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Equations for calibration curve generated from mean of four injections at each concentration

$^a$ huratoxin curve constructed from lower range of simplexin calibration curve (108.4 to 542 ng/ml, 4 points)

$^b$ acetoxyhuratoxin curve constructed from upper range of simplexin calibration curve (650.4 to 2710 ng/ml, 4 points)

Table 3.2 Concentration (µg/kg dry weight) of daphnane diterpene orthoesters detected in Phaleria nisidal extracts

<table>
<thead>
<tr>
<th>extract</th>
<th>simplexin (µg/g DW)</th>
<th>acetoxyhuratoxin (µg/g DW)$^a$</th>
<th>huratoxin (µg/g DW)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>lab MeOH</td>
<td>25.4 ± 4.0</td>
<td>113.5 ± 16.4</td>
<td>15.3 ± 2.4</td>
</tr>
<tr>
<td>lab H$_2$O</td>
<td>n.d.$^b$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>traditional</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>preparations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TB</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TT</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Obak</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TNgsar</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tnger</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

All values are expressed as means ± standard deviation of quadruplicate injections of three extraction replicates (based on TQD quantification SRM AUC relative to pure simplexin).

$^a$ Expressed as simplexin equivalent (semi-quantitative).

$^b$n.d., not detected; below the limit of detection.
3.3.2 Daphnanes in water extracts

Simplexin, huratoxin and acetoxyhuratoxin were not detected in the lab-prepared water extract or in traditional water infusions prepared by six healers (code: TI, TB, TT, Obak, TNgsar, Tnger) in Palau at our lowest limit of detection (30.4 ng/mL).

4. Discussion

4.1 Toxicity of daphnanes

Work detailed in the previous chapter resulted in the identification of several daphnane diterpene orthoesters as immunomodulatory compounds in *Phaleria nisidai*. These compounds are restricted to the Thymelaeaceae and Euphorbiaceae plant families and have unique structures as well as interesting bioactivities. This class of compounds has been shown to have anticancer, (Tranient receptor potential vanilloid 1) TRPV1-activating and cholesterol-lowering activities. In addition daphnanes possess irritant and tumor-promoting activities (Adolf et al., 1988; Hecker, 1985) and some plants containing these compounds are acutely harmful, and sometimes deadly, to livestock (Fletcher et al., 2009; Roberts et al., 1975; Wilson et al., 2007). While the effects observed in livestock and laboratory animals with the administration of simplexin and other daphnanes are severe, these toxic syndromes have yet to be displayed in humans.

Chapter 2 diverged from the traditional ethnomedicinal preparation of an aqueous extract because it was an exploratory untargeted metabolic study that required methanol to extract a broad range of metabolites. Aqueous solvents extract more polar compounds from a matrix (sugars, polar and glycosylated flavonoids) while the polarity of organic solvents, including methanol, is lower (Richardson and Harborne, 1985). Therefore more nonpolar compounds, such as steroids, fatty acids and di- and triterpenes, especially with extensive aliphatic moieties will be
extracted. In the previous chapter daphnanes were identified from nonpolar chromatographic fractions of the hexane partition of the methanol extract (See Chapter 2, Figure 2.3). The compounds have long (up to 11 carbon) saturated and unsaturated fatty acid esters attached to diterpene skeleta (Figure 2.10). It is not likely that these relatively nonpolar compounds would be extracted in water, but the fact that a plant containing at least six of these compounds is consumed routinely by humans in Palau is concerning considering their potential for toxicity and warrants further study.

Ethnographic results (Chapter 1 and Dahmer et al., 2012) indicate that fresh leaves are always boiled in water to make a tea; or leaves are used in topical steam baths or aromatherapy. The leaf is never ingested in whole form or as an alcohol extract, which could lead to exposure of diterpene intoxicants. Laboratory-prepared aqueous extracts as well as aqueous infusions prepared by local healers in Palau were analyzed for daphnane concentration and compared with the methanol extract.

These results indicate that simplexin is not present in water preparations above the 30 ng/mL limit of detection. The possibility remains that trace amounts of simplexin and other daphnanes are present in water extracts at amounts below our LOD. More sophisticated technology with higher quantitative sensitivity, such as quantitative NMR will need to be used to explore this hypothesis.

It is important to note that simplexin can exert biological activity in vivo at nanomolar and nanogram doses (Table 3.3). Blood and tissue samples were taken immediately after euthanization from cows that have been severely affected by St. George disease and no traces of simplexin were detected using a method able to detect simplexin to levels as low as 20 ng/mL (Fletcher et al., 2009). Simplexin acted as a growth inhibitor of murine lymphocytic leukemia at
5 ng/mL (Pettit et al., 1983), exerted anti-HIV activity in MT4 cells at 0.008 μM (4.256 ng/mL) (Asada et al., 2011) and inhibited a human sarcoma cell line at <0.1 μM (53.2 ng/mL) (Li et al., 2013). It has also shown irritancy when applied to mice at 0.03 nmoles/ear (Zayed et al., 1977) and acted as a tumor promoter when applied to mouse back skin at 20 nmoles (Adolf et al., 1988). It is difficult to extrapolate results of *in vitro* or animal *in vivo* studies to the human system, but it is clear that simplexin has *in vivo* activity at concentrations lower than what can be detected using highly sensitive instrumentation. If daphnanes are present in lower amounts that what can be detected using our LC-MS-TQD method Palauans may have discovered a low-dosage therapeutic window for daphnanes at which they exert beneficial immunostimulant activity while remaining non-toxic or cocarcinogenic.

**Table 3.3** Bioactivity and toxicity of simplexin at low doses reported from the literature and our experiment in Chapter 2. * indicates doses below our limit of detection of 30.4 ng/mL.

<table>
<thead>
<tr>
<th>Bioactivity</th>
<th>Dose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Inhibition of HIV-1 replication</td>
<td>*EC₉₀ = 8 nM (4.26 ng/mL)</td>
<td>Asada et al., 2011</td>
</tr>
<tr>
<td>Inhibited HT-1080 human sarcoma cell line</td>
<td>IC₅₀ = 0.1 μM (53.2 ng/mL)</td>
<td>Li et al., 2013</td>
</tr>
<tr>
<td>*Inhibited murine lymphocytic leukemia line</td>
<td>*EC₅₀ = 5 ng/mL</td>
<td>Pettit et al., 1983</td>
</tr>
<tr>
<td>Increase cytokine production</td>
<td>1 μg/mL</td>
<td>Chapter 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Dose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>*St. George’s disease (cattle)</td>
<td>*Not detected (LOD &lt; 20 ng/mL)</td>
<td>Fletcher et al., 2009</td>
</tr>
<tr>
<td>*Irritancy (mouse ear model)</td>
<td>*0.03 nmoles/ear (15.96 ng)</td>
<td>Zayed et al., 1977</td>
</tr>
<tr>
<td>Tumor promoter (mouse back skin)</td>
<td>20 nmoles (10.64 μg)</td>
<td>Adolf et al., 1988</td>
</tr>
</tbody>
</table>

These daphnanes have not shown any toxic effects in humans and it appears that the chronic consumption of *Phaleria nisidai* water infusions does not cause any acute or chronic disease. Most interview subjects did not warn of any side effects and this tea was often consumed constantly, in lieu of water, by people in Palau (Chapter 1, Table 1.3).
The possibility for bioaccumulation of daphnanes in fatty tissues may exist. The daphnanes in this study are lipophilic, coming from nonpolar fractions of a hexane partition of the crude methanolic *Phaleria nisidai* extract, with saturated and unsaturated aliphatic moieties attached to the orthoester. In humans lipophilic compounds move from the blood into hydrophobic regions of cell membranes. Compounds with a balance of lipophilicity and hydrophobicity move out of the membrane and into the cell, accumulating in fat deposits. Compounds with an octanol:water ratio of 0.1-1 are those that diffuse most readily across cell membranes (Walker et al., 2000). The partition coefficient (K<sub>ow</sub>) for simplexin, huratoxin and acetoxyhuratoxin has not yet been reported but could inform whether these compounds bioaccumulate in the human system, leading to chronic toxic effects.

To determine the etiology of St. George disease, a feeding trial was conducted by administering *Pimelea trichostachya* whole plants containing simplexin (approximately 200 ppm) at a dose of at least 7.5 mg dry plant/kg body weight/day to calves. Mild diarrhea was observed in the initial 4 day period, but no adverse effects were seen until day 35, when heart rates increased. Mild under-jaw edema was eventually observed but after 91 days the animals appeared normal through the end of the experiment at 125 days. The authors deduce that toxic effects diminished because the animals developed detoxification mechanisms through activation of liver enzymes or rumen bacteria (Fletcher et al., 2009). Further work remains to determine if chronic consumption of *Phaleria nisidai* has caused Palauans to develop similar detoxification mechanisms for simplexin.

In *Pimelea* species there are clear differences in distribution of simplexin throughout the plant. Seeds and flowers of *P. simplex* and *P. trichostachya* contain 11- and 14-fold more simplexin, respectively, than the leaves (Fletcher et al., 2009). In 26 interviews, not one person
reported using the reproductive structures of *Phaleria nisidai* for medicine or food, despite the distinctive bright red drupes that are produced. A hypothesis that remains to be tested is whether these organs are avoided because they are enriched in daphnane toxins.

4.2 *Food and medicinal plants with toxic concerns*

It is important to mimic the traditional preparation of botanicals with an ethnomedicinal history before any conclusions can be drawn about toxicity and activity as it pertains to the culture that is using the botanical. For example the "kava paradox" relates to the observation that hepatotoxicity related to the use of kava (*Piper methysticum*) is only rarely observed in traditional Pacific cultures while it is more frequently in Western users of the botanical (Currie and Clough, 2003; Moulds and Malani, 2003). These authors propose that in the West, kava is often administered as an alcohol or acetone extract, which has a much different chemical profile than the traditional aqueous cold water infusion. Recently, this hypothesis has been refuted as new clinical data emerge from Pacific countries reporting hepatotoxic effects associated with aqueous cold water infusions with aflatoxins present only in dried roots the latest culprit for observed toxicity (Teschke et al., 2011).

A similar case can be observed with the study of the traditional use of *Cinnamomum carolinense* medicinal infusions on Pohnpei. Bark shavings are brewed in hot water and consumed as a beverage or as a medicine to treat backache and joint pain. This plant contains saffrole, a known hepatotoxin, which is present in methanolic extracts. However, there is no hepatotoxicity observed in humans drinking hot water infusions of *C. carolinense* medicinally. It was observed that saffrole breaks down with the application of heat (Reynertson et al., 2005) implying that this culture developed a way to use this plant for its medicinal benefits while rendering its toxic components harmless.
Cassava, *Manihot esculenta*, is the second or third most important source of calories for 500 million people in Africa and Latin America (Cock, 1985). This is surprising considering that some cultivars of cassava are notorious for containing high amounts of toxic cyanogenic glycosides in its roots, the organ that is usually eaten. These compounds can cause acute toxicity, konzo, a type of paralysis in young people and ataxic neuropathy in old individuals, if too much is consumed (Vetter, 2000). WHO sets safe cyanogenic glycoside levels at 10 mg/kg flour (Cardoso et al., 2005). Cassava is made into "gari" by roasting the grated, dehydrated and fermented root tissue and this is the way most cassava is consumed in Nigeria (Okafor et al., 2002). This complicated processing method is critical, as it reduces toxin load by 90% (Maduagwu and Oben, 2007) though in some seasons this is not effective as environmental stresses can greatly increase glycoside content (Cardoso et al., 2005). As population increased and food resources became scarce, cassava was relied upon in Africa to provide energy and the toxin had to be overcome. Traditional knowledge once again found a solution to deal with a toxic plant, although in this case, improved methods of processing to eliminate, not simply reduce, cyanogenic glycosides is desired.

4.3 Active components of *Phaleria nisidai*

In the case of *Phaleria nisidai*, interview results indicate that alcohol is not used for medicinal extractions. Perhaps one reason for strict adherence to aqueous preparation of these leaves is due to the presence of potentially toxic daphnane esters that would be consumed in organic (such as alcoholic) preparations, that aqueous preparations minimize.

In Chapter 2 chemometric analysis of active fractions revealed that daphnanes were responsible for the activity of a *Phaleria nisidai* methanol extract. In this chapter daphnanes could not be detected in an aqueous extract therefore the compounds responsible for the activity
of traditional aqueous *P. nisidai* preparations remain to be determined. Mangiferin (Matsuda et al., 2004), the benzophenone iriflophenone-2-*O*-α-rhamnose and the flavonoid genkwanin-5-*O*-β-D-primeverose (Kitalong et al., 2012) and a mixture of acyglucosylsterols (Matsuda et al., 2005a) are the only compounds other than daphnanes that have been identified from this plant. Mangiferin, the most abundant compound in aqueous extracts, was inactive in an ELISA assay to measure IFNγ production by PBMCs (similar production to negative control) as well as in work done by Matsuda in *in vivo* and *in vitro* cytokine assays (Matsuda et al., 2004).

Matsuda's group did determine that a mixture of acylglucosylsterols (Matsuda et al., 2005a) was the active factor in a 50% EtOH extract of *Phaleria nisidai*. These compounds may be responsible for activity of the water extract in our study, however the sterol backbone and long hydrophobic fatty acid make these compounds quite nonpolar and thus not likely to be extracted in an aqueous infusion. In fact, a tentative identification of the acylglucosylsterol seen in Figure 3.4 was made from $^1$H and $^{13}$C NMR data of compound isolated from the hexane partition of a crude *Phaleria nisidai* methanol extract as part of this work. Due to difficulties with ionization in the LC-MS-ESI-TOF the tentative structure could not be confirmed because exact mass could not be obtained. The acylglucosylsterol found by Matsuda's group (2005b) was reported to have an exact mass of 837.6563 (calculated for [M+Na]$^+$ = C$_{51}$H$_{90}$O$_7$Na) by fast-atom bombardment mass spectrometry (LC-FAB-MS). We did not detect this mass, nor its protonated ion ([M+H]$^+$=C$_{51}$H$_{91}$O$_7$), in the water or methanol extracts of *P. nisidai* by electrospray ionization mass spectrometry (LC-ESI-MS) thus its presence, and contribution to activity, in methanol or water extracts cannot by confirmed by this work.

It is clear that mangiferin is a major component of water extracts (Kitalong et al., 2012b). As discussed in Chapter 1, mangiferin exerts effects on diverse biological systems, with
anti-tumor, anti-viral and CNS stimulatory (Pinto et al., 2005) activity. This compound is likely to play a role in the general activity of traditional aqueous preparations however its effects on the immune system tend to be more anti-inflammatory (Leiro et al., 2004) than the immunostimulatory responses shown in Chapter 2.

Figure 3.4 Acylglucosylsterol tentatively isolated and identified from hexane partition of $P. nisidai$, similar to compounds reported as having immunostimulatory activity by Matsuda's group (Matsuda et al., 2005a).

The next chapter will introduce the possibility that the plants we tested, although from a bulk sample from 27 trees, may have had lower amounts of daphnanes than some other populations of $Phaleria nisidai$ (Fletcher et al., 2009). Work remains to determine if trees containing high amounts of daphnanes as reported in Chapter 4 contain any of these compounds in their water extracts.
Chapter 4

Phytochemical variation of *Phaleria nisidai* in Palau

1. Introduction

1.1 *Phaleria nisidai* within Palau

Palau is a country comprised of 586 islands, all differing in habitat and levels of human settlement and disturbance. Geographic isolation and habitat diversity are expected to contribute to phenotypic variation within the organisms existing on these islands, making the existence of unique infraspecific chemical races likely. The largest islands in Palau are volcanic, but in terms of quantity, most of the Palauan islands are coral limestone "Rock Islands." These islands are covered by mature limestone forest, with a very high species diversity, despite very thin soil deposits consisting of decaying vegetation caught in the porous limestone substrate (Donnegan et al., 2007). Babeldaob is the largest island, and is volcanic in origin. Mangroves and swamps are common in lowland volcanic areas on Babeldaob and savanna and scrub savanna replaced thousands of acres of mature forest on this island as early as 2500 years ago as a result of anthropological disturbances such as terracing, which accelerated erosion of volcanic soil (Donnegan et al., 2007).

Remotely-sensed landcover data from Landsat 7 and Aster satellites was collected and classified, stratifying the land of Palau into different habitat types (Table 4.1). There was no habitat classification for Kayangel, but fieldwork conducted for this dissertation revealed it to be a low-lying atoll with sandy soil, represented by stands of coconut (*Cocos nucifera*) and assorted banana species (*Musa* spp.). A soil survey conducted by the USDA in 1980 classified Palau into 41 different soil types (Smith, 1980). These soil types were determined based on biological and mineral substrate, soil levels, drainage and slope and are each represented by characteristic flora.
Phytochemical variation is often observed between different populations of the same species, and this variation is often due to geographical differences (Moore et al., 2013). Differences in geography can comprise the cumulative effect of factors such as genetic drift, environmental pressures, herbivory and allelopathy and climatic variables on secondary metabolite production (Tetenyi, 1970). Chemical variants within a species are usually referred to as landraces, chemical races or chemotypes. Chemotype analysis can be employed to find the highest yielding populations of economically important chemicals (Johnson et al., 2008; Sandasi et al., 2012), locations likely to contain populations with the highest medicinal value (Laure et al., 2008; Seemann et al., 2010) or to assess metabolite production in response to geographic or environmental conditions (Milanowski et al., 2002; Sellami et al., 2013).

Due to the importance of *Phaleria nisidai* to the medicinal traditions of Palau this plant was chosen and metabolite concentrations across collection sites throughout these islands were compared. Relatively few phytochemical studies have been performed on this plant, with only mangiferin, the fatty acid α-tocopherol and a mixture of acylated glucosysterols (Matsuda et al., 2005a, 2004), the benzophenone glycoside iriflophenone 2-O-α-rhamnopyranoside and a
flavonoid; genkwanin 5-O-β-D-primeveroside (Kitalong et al., 2012a) previously identified by other groups (Figure 1.8). In Chapter 2 three daphnane orthoesters are reported from P. nisidai for the first time. These compounds; simplexin, acetoxyhuratoxin and huratoxin, possess interesting bioactivities typical of diterpene esters (discussed further in Chapter 3). They are potent protein kinase C (PKC) activators displaying anticancer and antiviral activities, but also have irritant and tumor-promoting activities (Liao et al., 2009). Simplexin is also responsible for acute GI inflammation in large mammals, leading to death if unmitigated (Fletcher et al., 2009; Seawright, 1984; Wilson et al., 2007), but this activity has not been displayed in human and non-human primates.

A quantitative study on mangiferin, simplexin, acetoxyhuratoxin and huratoxin (Figure 4.1) in over 200 trees was carried out using UPLC-MS-TQD. Daphnanes were chosen for this study because their potential toxicity, but also medicinal activity, make it crucial to understand natural variation in these compounds and if this has any relationship to human health in these islands. Mangiferin was also included in this study due its wide range of medicinal activity, including anti-diabetic potential (Fotie and Bohle, 2006; Im et al., 2009; Li et al., 2008). Most Palauans have a preferred collection site, and it is usually the closest tree to their home. They have not reported traveling to certain sites to collect Phaleria nisidai. This study will inform whether Palauans are exposed to different amounts of these medicinal and/or potentially toxic compounds.

1.2 Phytochemical variation in an environmental context

Habitat heterogeneity, combined with natural selection, often results in multiple, genetically and/or phenotypically distinct ecotypes within a single species (Hufford and Mazer, 2003). The term “chemotype” is often used in natural products research and refers to the
chemical phenotype, or chemical expression pattern, of a particular plant species (Vrhovsek et al., 2012). Therefore, a single plant species may have more than one chemotype. This chemotype can be observed by quantifying secondary metabolites and comparing them within species. Several studies have been published concerning the relationship of ecological variables and geographic location to levels of secondary metabolites and are presented in Table 4.2. Quantitative UPLC-MS-TQD was used in this study to determine if differences in secondary metabolites can be correlated to habitat, island or climatic variables at different population sites.

Figure 4.1 Compounds quantified by LC-TQD-MS. (1) simplexin, (2) acetoxyhuratoxin, (3) huratoxin and (4) mangiferin.

Other researchers have attempted to link phytochemical variation to geographic location or environmental variables present at collection sites. In the work by Seemann et al., (2010) Arnica montana plants were collected at 10 different sites and a set of ecological variables were extracted for each collection site. The levels of sesquiterpene lactones were determined by gas chromatography and were quantified both within population and between populations at different sites. ANOVA was performed to determine the similarity of collections within populations and
differences between populations. Univariate linear regression was performed, plotting the concentration of selected compounds against the range of values for each ecological variable. It was found that temperature and rainfall were correlated with total sesquiterpene lactone content and an individual sesquiterpene lactone, 6-O-isovalerylhelenaolin, was negatively correlated with temperature and positively correlated with rainfall and altitude. The authors performed principle components analysis (PCA) to investigate the differences in sesquiterpene lactone spectra between different habitat types. There were some differentiation trends between the Alp habitat and foothill habitat by phytochemical content but the principal components had a low discriminatory power and did not provide a clear separation.

Studies performed on *Podophyllum peltatum* (Moraes et al., 2005) found that total lignan content was correlated to light, and podophyllotoxin was influenced by soil pH. A map of populations containing high amounts of lignans was created to find the most valuable populations.

A species distribution model supported by strong statistical analysis was used to predict the location of high-artemisinin yielding *Artemesia annua* (Huang et al., 2010). Known locations of high quality *A. annua* were analyzed using GIS methods and the range of ecological factors for each location were analyzed using an ANOVA. The coefficient of variance (CV) for each variable was determined and variables with a low CV suggested that they could be a range-limiting factor for high quality *A. annua*, while factors with a high CV suggested otherwise. Other regions in China that had geographic locations for which ecological variables falling in the range of the low CV “limiting factors” were determined via GIS and chosen as potential high artemisinin yielding habitats. However these researchers did not confirm that predicted sites did in fact produce high quality *A. annua*. 
Table 4.2  Previous studies on the relationship of geography and ecology to phytochemistry

<table>
<thead>
<tr>
<th>Plant</th>
<th>Metabolites</th>
<th>Quantitative Methods</th>
<th>Ecological Variables</th>
<th>Sample size (n)</th>
<th>Statistical Methods</th>
<th>Outcome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Croton lechleri</td>
<td>Six benzylisoquinoline-derived alkaloids</td>
<td>HPLC-PDA by external standard</td>
<td>Location</td>
<td>15 field sites, 1 mature intact leaf collected from two individual branches, analyzed individually</td>
<td>2-way ANOVA</td>
<td>Geographic distribution of 3 chemotypes was mapped</td>
<td>(Milanowski, Winter et al. 2002)</td>
</tr>
<tr>
<td>Withania somnifera</td>
<td>14 withanolide steroids</td>
<td>HPLC-PDA by external standard</td>
<td>Populations from four locations were compared</td>
<td>For each plant, 2 extractions on 1g of leaf material carried out and analyzed separately</td>
<td>Comparison of averages with CV</td>
<td>3 ecotypes were found</td>
<td>(Bessalle and Lavie 1987)</td>
</tr>
<tr>
<td>Arnica montana</td>
<td>Total and 14 individual sesquiterpene lactones (SL)</td>
<td>GC-FID by internal standard, GC-MS for identification</td>
<td>Monthly rainfall, monthly temperature, altitude, soil C/N ratios, soil P and K contents</td>
<td>Same number of flowerheads collected from 6 locations in the 10 study sites</td>
<td>ANOVA, Univariate correlation, PCA</td>
<td>Temperature and rain influenced total SL content. One individual SL showed neg correlation to temp and pos correlation to rainfall and altitude</td>
<td>(Seemann, Wallner et al. 2010)</td>
</tr>
<tr>
<td>Pteridium arachnoid eum &amp; P. caudatum</td>
<td>Total phenolics (low molecular weight phenolics) and condensed tannins (high molecular weight phenolics)</td>
<td>Spectrophotometric</td>
<td>Altitude</td>
<td>8-12 samples of fully developed blades taken from each of 12 sites</td>
<td>Linear regression</td>
<td>Tannins were positively correlated with altitude</td>
<td>(Alonso-Amelot, Oliveros et al. 2004)</td>
</tr>
<tr>
<td>Podophyllum peltatum</td>
<td>Total and individual lignans, including podophyllotoxin</td>
<td>HPLC-PDA</td>
<td>Light; slope; elevation; temperature; precipitation; soil minerals, C, pH</td>
<td>21 whole plants collected from 5 regions. Within population sampling was performed at 2 locations, located &lt; 50m apart</td>
<td>Linear regression (least squares method)</td>
<td>More lignans from sunny locations, podophyllotoxin influenced by soil pH; map of valuable populations created</td>
<td>(Moraes, Momm et al. 2005)</td>
</tr>
<tr>
<td>Artemesia annua</td>
<td>Artemisinin</td>
<td>Not performed</td>
<td>Average monthly temperatures, annual sunshine time, total precipitation, relative humidity, altitude, soil type</td>
<td>180 accessions from Youyang County made for GIS analysis</td>
<td>ANOVA for species distribution modeling only</td>
<td>An predictive SDM was created to find other potential habitats for production of high quality A. annua</td>
<td>(Huang, Xie et al. 2010)</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.1 Plant Collection

In this study a population was considered to be a group of trees in a distinct geographical area. After the first tree was encountered the next tree within eyesight of the first tree was considered part of the population, followed by any trees within eyesight of the second tree, and so on. Every tree in a population was sampled and analyzed. Trees were not collected according to any particular transect or random sampling technique. Instead each field location inspected was traversed until Phaleria nisidai was encountered, or until it became more feasible to move to a different site. Many roadside stops, hikes to a ridge or Rock Island trips did not result in any P. nisidai populations.

Some populations consisted of only one tree, while others had up to 8 trees collected. Coordinates of the collection site were recorded by GPS. For each tree sampled, leaves were collected from median stem nodes of at least three different branches of various north, south, east and west orientations for a total of at least eight leaves per tree. Care was taken to not sample leaves with signs of herbivory or fungal growth, and very old and very young leaves (by visual inspection) were avoided. Leaf samples were dried in a wooden oven containing four 30W incandescent bulbs for 24 h. Voucher specimens were collected and deposited in the Belau National Museum, Natural History Section (Voucher numbers: DK001-DK034).

2.2 Habitat classification

Remotely-sensed data from Landsat 7 and Aster satellites were collected from 2001-2002. This raster data was classified using an unsupervised classification technique (Boucher, 2003). Classifications were groundtruthed in 2003 by Boucher and were verified again, during our 2011 fieldwork. There was no habitat classification for Kayangel, but fieldwork conducted
for this work revealed it to be a low-lying atoll with sandy soil, represented by stands of coconut
\((Cocos\ nucifera)\) and assorted banana species \((Musa\ spp.)\).

2.3 Soil types

A shapefile from the 1983 soil survey map produced by the USDA Soil Conservation Service was used to provide data on soil types for each population. No soil data was available for the three Rock Islands (Ongael, Ngeruktabel and 'Rock island 3') where collections were made.

2.4 Leaf processing

Eight dried leaves from each individual tree were stacked and a portion of leaf material weighing \(50 \pm 5\) mg was cut from the stack. This ensured that each leaf taken from an individual tree was represented and material from each leaf was collected from the same point along the lateral line of the leaf. Care was taken to avoid veins in the leaf cuts.

2.5 Extraction and sample preparation

The \(~50\) mg composite leaf sample from each tree was placed in a 50 mL glass centrifuge tube to which 25 mL of reagent grade MeOH was added. Samples were blended in the centrifuge tube to fine material with a handheld homogenizer and extracted under ultrasonic conditions for 30 minutes. After extraction, the tubes were centrifuged (3000 rpm x 10 min) and 5 mL of the supernatant was decanted and dried under N\(_2\) stream. Dried extracts were weighed and resuspended with LC/MS grade MeOH to 1 mg/mL concentrations. Extracts were passed through a .45 \(\mu\)m syringe filter before UPLC-MS-TQD analysis.

2.6 UPLC-MS-TQD analysis

Samples were analyzed by LC-MS/MS using a Waters Acquity UPLC (Waters Corp., Milford, MA) module. LC separation was conducted using a Waters Acquity UPLC Sample Manager coupled to a Waters Acquity Binary Solvent Manager. Separation was achieved on a 50
x 2.1 mm, 1.7 μm, Kinetex C18 (Phenomenex, Torrance, CA) column, held at a constant temperature of 40 °C. The mobile phase was composed of A, 0.1% formic acid in water and B, 0.1% formic acid in MeCN, at a flow of 0.5 mL/min with a gradient as follows: t₀, 80% A; t₀.5, 80% A; t₁.5, 20% A; t₂.5, 5% A; t₅, 5% A; t₆, 80% A; t₈, 80% A.

MS/MS detection was made using a Waters Acquity TQD tandem triple quadrupole mass spectrometer. Ionization was achieved using a multimode source in positive electrospray (ESI) mode at the following conditions: capillary voltage at 3.0 kV, cone voltage at 30 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 50 L/h, and desolvation gas flow of 500 L/h. The desolvation and cone temperatures were set at 450 and 150 °C, respectively. Argon was used as MS/MS collision gas with a flow rate of 0.15 mL/min and collision energy set to 20 eV.

2.7 Identification and quantification

Simplexin and mangiferin were positively identified based on MS/MS transitions and retention time comparison and coinjection with standard compounds. Relative retention times and the transitions seen in Table 4.3 were used to identify two additional daphnanes; huratoxin and acetoxyhuratoxin in methanol extracts. Calibration curves were constructed for mangiferin and simplexin, plotting the AUC of the most abundant daughter ion (mangiferin, m/z⁺= 273.1; simplexin, m/z⁺=253.3) vs. injected concentration. The calibration curve for mangiferin was constructed from five different concentrations (266-1419 μg/ml) injected in triplicate. Two calibration curves were used to quantify simplexin, acetoxyhuratoxin and huratoxin. A low-range curve was made from six points (72.96 to 912 ng/mL) injected in triplicate and an upper-range curve was prepared from 8 points (1.824 to 18.24 μg/mL) injected in triplicate. Huratoxin was
semi-quantitatively measured using the low-range curve for simplexin and acetoxyhuratoxin was semi-quantitatively measured using the upper-range simplexin curve.

Table 4.3 UPLS-TQD-MS parameters used for quantification of metabolites in this study.

<table>
<thead>
<tr>
<th>compound</th>
<th>RT</th>
<th>quantification SRM</th>
<th>confirmation SRM</th>
<th>LOD ng/mL</th>
<th>LOQ ng/mL</th>
<th>slope</th>
<th>intercept</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>simplexin</td>
<td>2.36</td>
<td>533.3 &gt; 253.3</td>
<td>533.3 &gt; 267.2</td>
<td>30.4</td>
<td>90</td>
<td>.3337</td>
<td>4.34</td>
<td>.999</td>
</tr>
<tr>
<td>huratoxin$^a$</td>
<td>2.87</td>
<td>585.5 &gt; 253.3</td>
<td>585.5 &gt; 267.2</td>
<td>304</td>
<td>90</td>
<td>.3337</td>
<td>4.34</td>
<td>.999</td>
</tr>
<tr>
<td>acetoxyhuratoxin$^b$</td>
<td>2.75</td>
<td>643.3 &gt; 207.2</td>
<td>643.3 &gt; 277.2</td>
<td>250.5</td>
<td>81.48</td>
<td>.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mangiferin$^c$</td>
<td>0.32</td>
<td>423.3 &gt; 273.1</td>
<td>423.3 &gt; 303.2</td>
<td>50</td>
<td>100</td>
<td>13.45</td>
<td>7282</td>
<td>.992</td>
</tr>
</tbody>
</table>

Equations for calibration curve generated from mean of three injections at each concentration

$a$ huratoxin curve constructed from lower range of simplexin calibration curve (108.4 to 542 ng/ml, 4 points)

$b$ acetoxyhuratoxin curve constructed from upper range of simplexin calibration curve (650.4 to 2710 ng/ml, 4 points)

$c$ two mangiferin curves were generated. High concentration curve: $y=13.45x + 7282$

Low concentration curve: $y=58.62x - 248.6$

2.8 Statistics

The Base R software package provided 'boxplot', 'aov', 'TukeyHSD' and 'cor' functions used for plotting, analysis of variance, comparison of means between sample groups and to provide correlation coefficients between variables, respectively (R Core Team, 2013). The 'doBy' package (Hojsgaard and Halekoh, 2013) provided the function 'summaryBy' and the 'psych' package (Revelle, 2013) provided the 'describeBy' functions used to obtain group means.

3. Results

3.1 Plant collection and environmental classification

Between March and July 2011 a total of 227 trees, representing 92 populations, were sampled from Palau. These populations were collected from ten different islands (Figure 4.3, Table 4.2). Remote-sensed data from the 2001 Landsat and 2002 ASTER landcover satellites were classified and groundtruthed in 2003. A map of Babeldaob with the habitat shapefile layered on top is shown in Figure 4.1. Table 4.4 shows the number of trees and populations and habitat types where populations were collected from each island. Not all islands were sampled equally. A map of all collected populations can be seen in Figure 4.3, Panel B.
Table 4.4. Number of populations and trees sampled from each island, and habitat types sampled from each island. BU: bare/urban; DF: degraded forest; HDF: highly degraded forest; Man: mangrove; MF: mature forest; Sav: savanna; ScSav: scrub savanna; nd: no habitat data; ng: populations not georeferenced; MLF: mature limestone forest

<table>
<thead>
<tr>
<th>Island</th>
<th>Populations (trees)</th>
<th>Habitat types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angaur</td>
<td>1 (3)</td>
<td>BU</td>
</tr>
<tr>
<td>Babeldaob</td>
<td>47 (113)</td>
<td>DF, HDF, Man, MF, Sav, ScSav</td>
</tr>
<tr>
<td>Kayangel</td>
<td>4 (4)</td>
<td>nd</td>
</tr>
<tr>
<td>Koror</td>
<td>3 (5)</td>
<td>ng, BU</td>
</tr>
<tr>
<td>Ongael</td>
<td>8 (27)</td>
<td>MLF</td>
</tr>
<tr>
<td>Malakal</td>
<td>1 (1)</td>
<td>BU</td>
</tr>
<tr>
<td>Meyuns</td>
<td>2 (4)</td>
<td>BU</td>
</tr>
<tr>
<td>Ngeruktabel</td>
<td>14 (47)</td>
<td>MLF</td>
</tr>
<tr>
<td>Peleliu</td>
<td>7 (11)</td>
<td>BU, MLF</td>
</tr>
<tr>
<td>Rock Island 3</td>
<td>5 (12)</td>
<td>MLF</td>
</tr>
</tbody>
</table>

Many islands did not have a diverse collection of habitats or on some islands, samples did not represent habitat diversity due to access problems. The island with the greatest number of sampled populations is Babeldaob (BA) containing 47 populations with 113 trees. Habitats were only compared within Babeldaob because of habitat homogeneity on other islands. For example, twenty-eight populations were collected from the rock islands, but they all represented mature limestone forest. Babeldaob was the only island that contained more than two sampled habitats.

3.2 Method validation

The LOD and LOQ were established using AOAC guidelines (AOAC International, 2013) as having a signal-noise ratio of at least 3:1 and 10:1. Simplexin had an LOD and LOQ of 30.4 and 90 ng/mL, respectively, and mangiferin had an LOD and LOQ of 50 ng/mL and 100 ng/mL, respectively.
Figure 4.2. Habitat classifications on Babeldaob based on Landsat (2001) and Aster (2002) remote-sensed ground cover data.
The repeatability of the analytical method was evaluated by analyzing the inter-day variation, measured as the relative standard deviation (RSD) of each analyte in a low- and high-daphnane extract. RSD is the standard deviation divided by the mean, also known as coefficient of variation, but referred to as RSD here to be consistent with AOAC terminology (AOAC, 2002). A low-daphnane extract (from tree 79A) had mean simplexin concentration of 169.95 μg/g with RSD of 12.1 and the high-daphnane extract (from tree 59E) had mean simplexin concentration of 1141.5 μg/g had an RSD of 14.1. Mangiferin from tree 79A had a mean concentration of 76.47 mg/g with an RSD of 4.08, while tree 59E contained mangiferin at 64.84 mg/g with an RSD of 14.8.

Extraction method reproducibility was established by making four extraction replicates. Slivers of eight leaves from a single tree were cut and pooled. Four 50 mg aliquots were weighed out. Each aliquot was extracted and injected three times. The RSD for all samples of simplexin was 19.5% and mangiferin was 18.8%.

Recovery was calculated by standard addition of 50 μg of the diterpene prostratin to a 50 mg leaf sample prior to extraction. Recovery of three replicate extractions injected four times each were between 90.1 and 115%.

3.3 Yields

The mean concentration and ranges of four compounds for all samples is shown in Table 4.5. A large amount of variation was observed and many trees collected did not contain any daphnanes above the LOD. Boxplots report central tendency as the median and are shown in Figure 4.3, Panel A. The median concentrations for all daphnanes is very low, while the mean is affected by the presence of a few outliers with large amounts of daphnanes. For example, simplexin and huratoxin each had a median of 0, while their mean concentration, which were
affected by the presence of high-yielding trees, were 88.9 and 82.4 μg/ml, respectively. This is also reflected by large standard deviations for these compounds. Mangiferin, had a more homogenous distribution than the daphnanes, and was detected in every tree with a median of 104.5 and a mean of 104.4 mg/ml, respectively, for all samples.

**Table 4.5 Central tendency and range of compounds quantified in this study.**
Mean shown as ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Simplexin (μg/g)</th>
<th>Acetoxyhuratoxin (μg/g)</th>
<th>Huratoxin (μg/g)</th>
<th>Mangiferin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>88.9 ± 224.6</td>
<td>785.4 ± 1725.7</td>
<td>82.4 ± 200.2</td>
<td>104.4 ± 75.8</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>0.0</td>
<td>110.7</td>
<td>0.0</td>
<td>104.5</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>2078.0</td>
<td>11584.0</td>
<td>1343.5</td>
<td>334.3</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

3.3.1. Daphnanes

To determine patterns in daphnane concentration, populations were grouped by island and compared (Figure 4.3, Panel A). Visual observation of boxplots indicated that Babeldaob contained outlying populations with very high amounts of daphnanes. These outlying populations had an effect on the mean, with mean concentrations of simplexin, acetoxyhuratoxin and huratoxin higher than all other islands at 173.0, 1444.9 and 160.4 μg/ml, respectively (Table 4.5). Other islands, including three rock islands, had very low mean daphnane quantities (Table 4.6) and did not have high-yielding outliers (Figure 4.3).

Populations from Babeldaob were then isolated and analyzed for intra-island differences in compound concentration. Only Babeldaob was selected for this analysis because it was the only island that had populations collected from different habitats. Boxplots, in Figure 4.4, show that savanna and scrub savanna contain higher amounts of daphnanes than all other habitat types. Tukey HSD test for multiple comparisons confirms that these two populations contain significantly higher amounts (p<0.05) of each individual daphnanes than all others.
Due to the wide range of variation within islands and habitats it cannot be confirmed that island or habitat alone contributes to high amounts of daphnanes. Outliers seen in the boxplot are informative because they indicate the presence of trees with a very high daphnane content.

A correlation determination between daphnane concentration and 19 continuous Worldclim bioclimatic variables (Hijmans et al., 2005) as well as elevation was made. No correlations were observed between simplexin, acetoxyhuratoxin, huratoxin, or total daphnanes with any of these variables. These variables are listed in Table 4.7.

Populations were also grouped according to soil type (Smith, 1980); however the variation within each soil type was so great that it is not likely to contribute to observable differences in daphnane concentration (Figure 4.6).

**Table 4.6** Average amount of daphnanes and mangiferin by island. Mean shown as ± standard error.

<table>
<thead>
<tr>
<th>Island</th>
<th># samples</th>
<th>simplexin (μg/g)</th>
<th>acetoxyhuratoxin (μg/g)</th>
<th>huratoxin (μg/g)</th>
<th>mangiferin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angaur</td>
<td>3</td>
<td>8.5 ± 3.2</td>
<td>158.2 ± 47.8</td>
<td>9.2 ± 4.4</td>
<td>50.7 ± 5.7</td>
</tr>
<tr>
<td>Babeldao</td>
<td>113</td>
<td>173.0 ± 13.4</td>
<td>1444.9 ± 105.6</td>
<td>160.4 ± 12.3</td>
<td>156.7 ± 2.5</td>
</tr>
<tr>
<td>Kayangel</td>
<td>4</td>
<td>7.5 ± 2.1</td>
<td>432.4 ± 111.3</td>
<td>39.4 ± 7.5</td>
<td>195.7 ± 10.9</td>
</tr>
<tr>
<td>Koror</td>
<td>5</td>
<td>30.8 ± 9.7</td>
<td>481.3 ± 95.1</td>
<td>14.0 ± 6.9</td>
<td>132.4 ± 3.7</td>
</tr>
<tr>
<td>Ongael</td>
<td>27</td>
<td>0.4 ± 0.2</td>
<td>18.0 ± 2.7</td>
<td>0.0 ± 0.00</td>
<td>30.1 ± 2.1</td>
</tr>
<tr>
<td>Malakal</td>
<td>1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.00</td>
<td>239.0 ± 13.4</td>
</tr>
<tr>
<td>Meyuns</td>
<td>4</td>
<td>13.9 ± 2.7</td>
<td>104.8 ± 19.2</td>
<td>26.7 ± 6.4</td>
<td>125.4 ± 19.3</td>
</tr>
<tr>
<td>Ngeruktabel</td>
<td>47</td>
<td>4.24 ± 0.8</td>
<td>120.3 ± 20.8</td>
<td>2.7 ± 0.7</td>
<td>25.5 ± 1.2</td>
</tr>
<tr>
<td>Peleliu</td>
<td>11</td>
<td>0.0 ± 0.0</td>
<td>60.6 ± 14.5</td>
<td>1.0 ± 1.0</td>
<td>125.9 ± 6.3</td>
</tr>
<tr>
<td>Rock Island 3</td>
<td>12</td>
<td>12.2 ± 3.3</td>
<td>265.9 ± 59.3</td>
<td>7.0 ± 2.5</td>
<td>21.4 ± 1.9</td>
</tr>
</tbody>
</table>


Figure 4.3. A: Boxplot for daphnanes (A1-3) and mangiferin (A4) grouped by island. Thick horizontal line of box indicates median; upper and lower lines indicate 75% and 25% quartiles (interquartile range, IQR) respectively. Whisker represents 1.5xIQR and circles represent outliers above this value. B: Map showing location of 91 populations of Phaleria nisidai collected on 10 islands of Palau.
**Figure 4.4.** Boxplot for daphnanes grouped by landsat classification within Babeldaob. Thick horizontal line of box indicates median; upper and lower lines indicate 75% and 25% quartiles (interquartile range, IQR) respectively. Whisker represents 1.5xIQR and circles represent outliers above this value. Width of box corresponds to number of samples included in each habitat category. DF: degraded forest; HDF: highly degraded forest; Man: mangrove; MF: mature forest; Sav: savanna; ScSav: scrub savanna.
Table 4.7 Worldclim variables.

<table>
<thead>
<tr>
<th>Worldclim Code</th>
<th>Variable</th>
<th>Worldclim Code</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio1</td>
<td>annual mean temp</td>
<td>Bio11</td>
<td>mean temp coldest quarter</td>
</tr>
<tr>
<td>Bio2</td>
<td>mean diurnal temp range</td>
<td>Bio12</td>
<td>annual precipitation</td>
</tr>
<tr>
<td>Bio3</td>
<td>isothermality (Bio2/Bio7)*100</td>
<td>Bio13</td>
<td>precip of wettest month</td>
</tr>
<tr>
<td>Bio4</td>
<td>temperature seasonality</td>
<td>Bio14</td>
<td>precip of driest month</td>
</tr>
<tr>
<td></td>
<td>(standard deviation Bio1)*100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bio5</td>
<td>max temp of warmest month</td>
<td>Bio15</td>
<td>precip seasonality</td>
</tr>
<tr>
<td>Bio6</td>
<td>min temp of coldest month</td>
<td>Bio16</td>
<td>precip of wettest quarter</td>
</tr>
<tr>
<td>Bio7</td>
<td>temperature annual range</td>
<td>Bio17</td>
<td>precip of driest quarter</td>
</tr>
<tr>
<td>Bio8</td>
<td>mean temp wettest quarter</td>
<td>Bio18</td>
<td>precip of warmest quarter</td>
</tr>
<tr>
<td>Bio9</td>
<td>mean temp driest quarter</td>
<td>Bio19</td>
<td>precip of coldest quarter</td>
</tr>
<tr>
<td>Bio10</td>
<td>mean temp warmest quarter</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 Mangiferin

The distribution of mangiferin by island has a different pattern than that observed for daphnanes. Babeldaob contained samples with the highest-yields of mangiferin, but the greatest median mangiferin concentration came from Malakal and Kayangel (Figure 4.3, Panel A). When comparing means Malakal (239.0 mg/g) and Kayangel (195.7 mg/g) contain more mangiferin than any other island, however both of these islands were not sampled extensively, each represented by only one population. Populations from the three rock islands and Angaur contained significantly lower amounts of mangiferin than all other islands.

Figure 4.5. Boxplot of mangiferin by habitat type. Width of box is relative to amount of samples included in each habitat category. BU: bare/urban; DF: degraded forest; HDF: highly degraded forest; Man: mangrove; MF: mature forest; MLF: mature limestone forest; Sand: sandy atoll; ng: not georeferenced; Sav: savanna; ScSav: scrub savanna
Because mangiferin level was variable in each of 10 islands, populations from all islands, not just Babeldaob, were grouped according to habitat (Figure 4.5). Sandy atoll, highly degraded forest, mature forest and scrub savanna were ranked as the habitats with highest mangiferin concentration. Sandy atoll (4 populations, 4 trees, mean=195.7 mg/g) and highly degraded forest (3 populations, 7 trees, mean=193.3 mg/g) had the highest mean concentrations of mangiferin. Mangroves (1 population, 1 tree, mean=44.6 mg/g) and mature limestone forest (28 populations, 87 trees, mean=27.2 mg/g) had the lowest mean concentrations of mangiferin, significantly different than all other habitat classifications, which is expected due to low-yielding populations observed for all rock islands.

A correlation analysis between mangiferin concentration and 19 continuous Worldclim bioclimatic variables (Hijmans et al., 2005) as well as elevation was conducted. Mangiferin is positively correlated with bio15 ($R^2=0.41$) and negatively correlated with bio14 and bio17 ($R^2=0.53$, $R^2=0.47$; respectively). Bio15 is precipitation seasonality, bio14 is precipitation in driest month and bio17 is precipitation of driest quarter. In locations with less precipitation (in the driest quarter or month) there is greater accumulation of mangiferin. These variables are all correlated with each other (bio14~bio15: $R^2=0.62$; bio14~bio17: $R^2=0.98$; bio15~bio17: $R^2=0.58$), so it is difficult to determine exactly which variable has the greatest effect, but this data indicates that precipitation may play a role in mangiferin production.

Populations were also grouped according to soil type (Smith, 1980) however the variation within each soil type was so great that it is difficult to determine if any particular soil type contributes to mangiferin concentration (Figure 4.6).
4. Discussion

4.1 Daphnane variation

The results presented in this chapter indicate that the concentration of daphnanes in *Phaleria nisidai* in Palau is highly variable. A median of zero indicates that most of the 227 trees sampled did not contain any simplexin; however UPLC samples of 108 trees did contain this compound above the lower limit of detection (30 ng/mL). As seen in Figure 4.1, trees containing appreciable amounts of daphnanes were only found growing on Babeldaob. The rock islands were sampled extensively, providing 27 populations and 86 trees. It was hypothesized that rock island populations would have more daphnanes due to the high salt levels, lack of soil and exposure to high winds that characterize the limestone Rock Islands, however Ngeruktabel, Ongael and 'Rock Island 3' each had mean simplexin levels below 12.2 μg/g.
Figure 4.7. Populations in the upper quartile for daphnanes and mangiferin, represented by red stars and green triangles, respectively. Populations in lower three quartiles represented by black dots.

Twenty-eight trees contained high amounts of simplexin, at levels above 200 ppm (200 μg/g). *Pimelea trichostachya* plants containing about 200 ppm simplexin (Fletcher et al., 2009)
are responsible for acute toxicity and chronic symptoms characteristic of St. George disease, which sometimes leads to death, in grazing animals in Australia. An extrapolation would assume that if sheep, cattle and horses were grazing on the leaves of these *Phaleria nisidai* populations St. George disease would develop thus it is surprising that people in Palau brew a medicinal tea from this plant, with some drinking it regularly, in lieu of water. However, Palauans are not eating *P.nisidai* leaves or making alcoholic tinctures and in Chapter 2 it is observed that the traditional aqueous infusion does not extract simplexin and other daphnanes, minimizing exposure to potentially toxic compounds.

The population containing the highest amount of simplexin was population 6, with mean content of 859.0 μg/g. This is a wild population existing in a mature forest habitat in Babeldaob. Population 89 has the highest total daphnane content, with mean content of 9.12 mg/g. Populations in the upper quartile (above 1.6 mg/g) for total daphnanes are shown in Figure 4.7. All of these locations are in Babeldaob as no other islands had populations that produced appreciable amounts of daphnanes. Three of the twelve populations in the upper quartile for simplexin (22, 46 and 49) existed in home gardens. Special attention should be paid to users of these home garden *Phaleria nisidai* trees. Often the same home garden tree is used for generations. It would be a worthy investigation to observe if current Palauans, or their relatives, using these particular trees have displayed any symptoms of toxicity or if they have any extraordinary uses or testimonials regarding this medicine.

4.2 *Mangiferin variation*

According to our work, mangiferin is the metabolite present in the largest abundance in *Phaleria nisidai* (mean for all population: 104.4 mg/g dry weight). As a relatively polar compound, mangiferin dissolves well in water (Matkowski et al., 2013) making it relevant to the
traditional use and preparation of this plant. Mangiferin possesses a range of bioactivities (Fotie and Bohle, 2006), and one relevant to its traditional therapeutic use is against diabetes. It has shown blood-glucose lowering properties in mice (Matsuda et al., 2004; Miura et al., 2001), ability to increase insulin sensitivity (Miura et al., 2001), and improve oral glucose tolerance (Muruganandan et al., 2005).

Results from this work indicate that mangiferin concentration has a wide variation, and if *Phaleria nisidai* is to be used in a clinical treatment for diabetes, as is currently planned by our colleagues at the Ministry of Health and the Palau National Hospital (Christopher Kitalong, personal communication, 2013), the levels of mangiferin should be measured in every batch of medicinal tea produced for this treatment. Babeldaob, Meyuns, Koror and Malakal populations all contain high-mangiferin samples so it is not necessary to travel to distant islands to sample high-mangiferin phenotypes. In fact, Rock Island samples contained the lowest concentrations of mangiferin, with a mean of 26.4 mg/g.

Because Rock Islands had the lowest mangiferin containing samples, it is expected that the mature limestone habitat also has the lowest amount of mangiferin (27.2 mg/g). All other habitats, besides mangroves, had a mean concentration above 121.8 mg/g. Variation of mangiferin in these habitats is high thus it is difficult to determine which of these habitats correlates most with high mangiferin concentration.

4.3 Relationship between mangiferin and daphnanes

The level of all three daphnanes is similar within populations, however mangiferin does not have the same expression patterns as the daphnanes. The highest correlation exists between acetoxyhuratoxin and huratoxin ($R^2=0.77$), and simplexin and huratoxin ($R^2=0.77$). There was smaller correlation between simplexin and acetoxyhuratoxin ($R^2=0.64$). Mangiferin had a very
low correlation with each daphnane \((R^2<0.20)\). Mangiferin is produced from a different biosynthetic pathway than daphnanes, and it is expected that two the types of compounds will not be correlated. Results indicate that these two pathways are affected differently by the same external factors. The boxplots (Figs 4.3-4.6) make it clear that expression levels for mangiferin and daphnanes differs for the same island, habitat or soil classification.

Populations in the upper quartile for mangiferin and total daphnanes are mapped in Figure 4.7, these populations contained higher concentrations than 75% of all populations. Upper quartile populations were mapped and compared with the remaining populations to determine if a geographic pattern could be observed for high-yielding populations. The same population exists in the highest quartile for both mangiferin and daphnanes in only 3 out of 21 populations, which could be expected based on their weak correlation. Also, of note on this map, many populations in the lower three quartiles exist very closely to upper quartile populations. There does not appear to a geographic relationship to populations enriched in these compounds but proper spatial analysis, such as Mantel's test, needs to be performed to confirm this assumption.

4.4 Conclusions

Two questions asked to every interview participant was "Where do you collect delal a kar?" and "Is delal a kar better when collected from specific places?" to determine if there was a geographic bias to Phaleria nisidai collection. Every informant replied that there is no difference in P. nisidai when collected from different areas. They were content to collect from home gardens, neighborhood trees, or wild trees near their house. In no interviews did people report traveling to the Rock Islands for collection of P. nisidai. As these populations contained the lowest amounts of mangiferin and daphnanes, their lack of travel to the Rock Islands to collect these plants is justified. This is opposed to the collection of Aidia racemosa (Cavanilles)
Tirvengadum (kerumes) where men will travel to the rock islands for the sole purpose of collecting kerumes to make a tea for virility (Dahmer et al., 2012).

The genetic relationship of these collections has not been evaluated. The presence of different chemical genotypes may be influencing the variation in chemistry observed in this study. These chemotypes may represent genetic varieties or subspecies that cannot be distinguished visually. Leaf material from each tree in this study is preserved for future genetic analysis. Age of tree, herbivory, diurnal cycle and lunar cycle are other factors that are not accounted for in this study. Future common garden transplant experiments should be performed to help determine further variables that may contribute to secondary metabolite accumulation in Phaleria nisidai.

It is important to locate trees containing high amounts of these compounds because mangiferin and daphnanes are compounds with pharmacological activities relevant the traditional use of Phaleria nisidai. If variables that contribute to differences in these secondary metabolites are identified they can be monitored or manipulated in future ethnopharmacological and clinical studies to take place in Palau.
Chapter 5

Conclusions

Traditional knowledge is the knowledgebase attained by a culture over hundreds of years through contact with their environment and usually includes knowledge of plants, animals and natural phenomena (Inglis, 1993) that a culture can utilize and manage for survival. A common view in Palau, observed through these interviews, and in the Minister of Health's foreword to the PPHCM (Dahmer et al., 2012) is that traditional knowledge is diminishing as time progresses. This pattern is observed for many different cultures throughout the world as modernization is thought to decrease the knowledge humans have of living things (Wolf and Medin, 2001). Knowledge of local flora, food crops and food preparation, natural climatic patterns, construction, and hunting and fishing techniques have all been observed to be decreasing in various cultures (Balick, 2003). As Balick points out, "Concern for the loss of traditional knowledge is the driving force behind many of the ethnobotanical and culture-related projects now underway throughout the world." Indeed that is a primary reason why the Palau Primary Health Care Manual was published and why the research performed in this dissertation was undertaken.

One example of an ethnobotanical project taking a novel approach to preserving traditional knowledge is the work on prostratin by Paul Cox and the Institute for Ethnomedicine. Prostratin was isolated from Homalanthus nutans during an National Cancer Institute (NCI) screening program to identify anticancer and anti-HIV compounds (Gustafson et al., 1992) and shows promising anti-viral activity. This plant was used by traditional healers in Samoa to treat hepatitis and ethnobotanical work conducted by Cox established prior knowledge of the use of this plant by Samoan healers. The Falealupo covenant was drafted and signed, which gives 20%
of all profits from the development of prostratin to the people of Samoa. Because of this contract, by 2001, almost US$ 500,000 has been provided to Falealupo village where the use of *Homalanathus nutans* was first demonstrated. This money has gone to medical clinics, supplies for clean water, schools and an endowment for rain forest protection (Cox, 2001). As a result of this income villagers have been able to resist pressures from logging companies looking to pay for rights to the surrounding forest. This is an example of bioprospecting done right, where ethnobotanical studies are linked to relevant, modern biomedical assays but the cultural source of the promising drug is not forgotten. This exemplifies the benefits that can be obtained to people of both traditional and Western cultures when there is an environment of trust and sharing of benefits.

This dissertation work was only realized because of the hard work put in to develop trust between The New York Botanical Garden and anthropologists, researchers and healers in Palau. The research highlights of this dissertation demonstrate potential benefits for the people of Palau. In Chapter 2 it is revealed that daphnane diterpene esters contribute to the immunostimulatory activity of *Phaleria nisidai* extracts. Another diterpene ester, prostratin, shared structural similarities to classic protein kinase C activators (Gustafson et al., 1992) such as TPA and the National Cancer Institute was close to halting further research with this compound (Cox, 2001) because it resembled phorbol tumor promoters. It was argued that "the contemporary ethnobotanical profile and the long history of use by the Samoan people indicated that it was not acutely toxic as were other phorbol esters." Research resumed and prostratin was discovered to be a promoter of HIV replication in T-cells acting as latent reservoirs of the virus, a final hurdle to eradication of the disease (Kulkosky et al., 2001).
Parallels can be drawn between prostratin in *Homalanthus nutans* and simplexin in *Phaleria nisidai*. *P. nisidai* certainly has a long history of sustained regular use, with very few acute or chronic toxic events reported. Similarly, it appears as though research into the pharmacological profile of simplexin has dried up. Despite showing promise as an anticancer agent no new bioactivity, structure-activity or mechanistic work has appeared in scholarly literature since 1983 (Pettit et al., 1983), with the exception of one feeding study in cattle (Fletcher et al., 2009) Perhaps this is due to its mild activity as a cocarcinogen in classic mouse models of ear irritation and back skin tumors (Zayed et al., 1982). The sustained use of a simplexin-containing plant in Palau may indicate that a therapeutic window exists where the beneficial properties of simplexin and related diterpene esters are exploited while their toxic effects are minimized.

Chapter 3 demonstrates that although daphnanes are present in small amounts in active nonpolar fractions and methanolic extracts of *Phaleria nisidai* leaves they cannot be detected in water extracts, the predominant preparation of this plant in Palau. It needs to be highlighted that these compounds are active at very low concentrations (see Discussion, Chapter 3) so the potential for long-term chronic toxicity cannot be ruled out. Medical professionals in Palau should be made aware of the potential for toxicity so they can carefully observe side effects, and causes of mortality in people taking this tea in large quantities to see if public health interventions need to be made. It is also possible that the water extract provides a critical low dose of daphnanes that is effective in immune stimulation without causing toxicity, or that other compounds present in the water extract are protecting Palauans from detrimental effects.
Chapter 4 has identified populations that contain daphnanes in relatively higher quantities than the average population. Individuals who are using these particular populations to produce *Phaleria nisidai* tea should be observed ethnobotanically and diagnostically. Do they have special uses, preparations or precautions that others do not have? Quantification of the nontoxic xanthone mangiferin was also performed. People using these populations as a source of medicine should be queried about their specific use patterns, in order to gain more insight into the biological activity of mangiferin in humans. Information on biological variation will also be informative to the observational ethnopharmacology study currently planned through the Belau National Hospital, in which patients with diabetes who are already drinking this tea as treatment are clinically monitored (Christopher Kitalong, personal communication, 2013; C Kitalong et al., 2012).

This project was initiated to contribute to the scientific support of traditional medicine, thereby validating the traditional knowledgebase this system is derived from. Evaluating traditional medicine can also contribute to treatment decisions made by doctors, healers and laypeople in Palau. It is the author's hope that positive results will contribute to the preservation of this system of healing by garnering respect for the Palauan pharmacopoeia in Palau, but also by the conventional scientific establishment outside of Palau. This work has also opened up doors for further inquiry into Palauan traditional medicine, biological effects of daphnanes and mangiferin and active components of traditional Palauan *Phaleria nisidai* preparations. The author believes that agreements such as that made between Dr. Paul Cox, the AIDS Research Alliance and the people of Samoa (Cox, 2001) should be consulted by leaders in Palau, as this can add value to their natural resources while providing motivation for younger generations in Palau to learn about this unique, endemic form of traditional medicine.
Appendix A

Questionnaire used for structured interviews

Name: Birthplace: Age:

Delal a Kar Questionnaire

1. Do you use delal a kar?
2. What do you use delal a kar for? If you do not use it, what do others use it for?
3. How many times have you used delal a kar in the past year?
4. How is delal a kar prepared?
5. What parts of the tree do you use? (any particular leaves)
6. Is delal a kar used alone, or in combination with other plants?
7. Where do you collect delal a kar?
8. Is delal a kar better when collected from certain locations than others?
9. Do you collect delal a kar at a certain time, such as when flowering or not?
10. Do you use the fruit or flowers?
11. Do you have any special ways of taking care of delal a kar or ways to help it to grow?
12. Are there ways to make delal a kar stronger?
13. Are there any times when you should not use delal a kar?
14. Who has taught you how to prepare and use delal a kar?
15. Who in your family uses delal a kar?
16. Do you live in the same place you were born?
17. Do you grow your own food? Where do you obtain most of your food?
18. Do you go to the hospital or doctor when sick? What is the first thing you do when you are sick or injured?
19. Is there anything you would like to know about delal a kar?
Appendix B

Informed consent forms
Appendix B.1
Page 1 of the informed consent form, informing participants of this research and that their traditional knowledge will be protected by a materials transfer agreement.

**INFORMED CONSENT FORM**

My name is Daniel Kulakowski and I am student in the Biology Ph.D. Program at The Graduate Center of the City University of New York (CUNY), working in collaboration with Christopher Kitalong, the Belau National Museum and the New York Botanical Garden (NYBG) on this project, entitled “Ethnomedicinal Assessment of Delal a kar - Phaleria nisidai.” This is a research study on the use, collection and management strategies of delal a kar. The study is expected to help conserve traditional knowledge in Palau and inform doctors about the ways in which Palauans use traditional medicine. I would like permission to interview you about your experiences with delal a kar.

This survey is part of the Ethnobotany of Palau Project that has as its goal the study of local traditional culture as it relates to the uses of plants for many purposes, including food and medicine, with the goal of conservation of this knowledge, along with the biodiversity of the natural environment that it is based upon. This research was initiated and is sponsored by The New York Botanical Garden.

The interview should take approximately 15-20 minutes. With your permission, I would like to videotape this interview so I can record the details accurately. The tapes will only be heard by my advisors and I. All information gathered will be kept strictly confidential, and will be stored in a locked file cabinet, to which only I, and my advisor, will have access. At any time you can refuse to answer any questions or end this interview.

The risks from participating in this study are no more than encountered in everyday life. The benefit of your participation is that you will help add to the preservation of traditional medicinal knowledge in Palau, for future generations to reference, and also to provide information that will ultimately help physicians treat community members who are using this medicinal plant. There will be approximately 100 participants taking part in this study.

I may publish results of the study, but names of people, or any identifying characteristics, will not be used in any of the publications. If you would like a copy of the study, please provide me with your address and I will send you a copy in the future. The results of this study are strictly academic and no profits will be made without the permission of the research participants. The intellectual property rights of participants in this study are protected by a material transfers agreement between Christopher Kitalong and CUNY (Docket #: TCO-09B5012-MTA).

If you have any questions about this research, you can contact me at dmkula@gmail.com, or my advisor Dr. Michael J. Balick at mbalick@nybg.org. If you have questions about your rights as a participant in this study, you can contact Kay Powell, IRB Administrator, The Graduate Center, the City University of New York, (212) 817-7525, kpowell@gc.cuny.edu.

We would greatly appreciate and value your contribution to this survey. I will give you a copy of this form to take with you.

Thank you very much.
Appendix B.2
Page 2 of informed consent form from Palau Bureau of Arts and Culture. Participants were asked to sign this page.

Ethnographic Assessment of Dehla Kar

(Project Name)
Through the Bureau of Arts and Culture

Kengei or a Ngemil a Tekoi me a Siasing (Oral History/Ethnography Agreement)

Njiek (Name) ___________________________ or a (State) ___________________________

Ngak a kongei el mo mengai a chulchedechel xel mo er a mesil el record er a chelcheh el __________ el kebessanigil el
el buiah er a ruk er a __________ el obengterir a reccheda a me ng mo sebechel el oubech aloka el tekoi me a chulchededuch
el ngar el alka el tape me a video, me a siasing el mo usbechel er a tekoi er a omesuub me a omengeluub el tekoi er a
Kechibelau el mo utulri el a kongei er a merred er er a me el tir a oureor el merritil el mengeluub el tekoi el kirel a
ulcheddu el Kiechibelau. A Ohsa er a Ibetel a Cherechar (Bureau of Arts and Culture) a kongel el mo mai el ottralka a
roku el tebibi el kirel a ulkerreul el me a mekelel el a usbech el alka el tekoi el ngar el a tape, video, me a siasing.

Ng dirrek el sebechem el imis er a kor el diak el soam el onger er ngii; tarebengil a chulchededuch er a ngii di el
lame; e subedii a chad el mengeduveduch er kau a iskum en ng ngar er ngii a chulchedecham el diak el soam a memengai
el mo er a tape.

Chelcheh el sel kimuchles a rokuel tekoi el kiram el meluches el mo merek, e ak imut el me el mo chamului el
roku el leko er kau me ng sebechem el mo medengel el kmo a chulchedecham el kullai ng ungii el diak a ko er a chelchehid
er ngii?

I agree to participate in a recorded interview including photograph-taking on __________ (date)

with __________ (Researcher Name)

David Kubekoski

agrees to honor any and all written and stated restrictions on
the use of this interview information.

Please know that: You may skip any question you prefer; stop the interview at any time; or choose to keep part of the
interview confidential by informing the interviewer that the portion of the information is confidential.

A iskum en ng ngar er ngii a kerim me a lechub en ng di soam el meleko el kirel tia el ureor e
momekedong el BAC, 488-2489.

If you have any questions or concerns about this research project, please contact the Bureau of Arts and
Culture at 488-2489.

In Agreement:

Saing (Signature of Interviewee) / Date ___________________________ Saing (Signature of Interviewer) / Date ___________________________
Appendix C
IRB Approval and Materials Transfer Agreements
Appendix C.1
CUNY Institutional Review Board Approval Letter for Ethnographic Interviews

Office of the Vice President for Research and Sponsored Programs
Committee on the Protection of Human Subjects

The Graduate School and University Center
The City University of New York
365 Fifth Avenue
New York, NY 10016-4309
TEL: 212.817.7623 FAX: 212.817.1329

TO: Mr. Daniel Kulakowski
Biology

FROM: Richard G. Schwartz, Ph.D.
Graduate Center IRB

SUBJECT: IRB Approval (Expedited Review)

STUDY: 11-01-019-0135 Ethnobotanical Assessment of Phaleria nisidai

DATE: March 24, 2011

The Graduate Center IRB has approved the above study involving humans as research subjects. This study was Approved - Expedited Category: 6 & 7 - based on 45CFR46.

IRB Number: 11-01-019-0135 This number is a Graduate Center IRB number that should be used on all consent forms and correspondence.

Approval Date: March 24, 2011
Expiration Date: March 23, 2012

This approval is for a period of one-year or less. You should receive a courtesy renewal notice before the expiration of this project’s approval. However, it is your responsibility to insure that an application for continuing review approval has been submitted before the expiration date noted above. If you do not receive approval before the expiration date, all study activities must stop until you receive a new approval letter. There will be no exceptions. In addition, you are required to submit a final report of findings at the completion of the project.

Consent Form: All research subjects must use the approved and stamped consent form. You are responsible for maintaining signed consent forms for each research subject for a period of at least three years after study completion.

Mandatory Reporting to the IRB: The principal investigator must report, within five business days, any serious problem, adverse effect, or outcome that occurs with frequency or degree of severity greater than that anticipated. In addition, the principal investigator must report any event or series of events that prompt the temporary or permanent suspension of a research project involving human subjects or any deviations from the approved protocol.
Amendments/Modifications: All amendments/modifications of protocols involving human subjects must have prior IRB approval, except those involving the prevention of immediate harm to a subject. Amendments/modifications for the prevention of immediate harm to a subject must be reported within 24 hours to the IRB.

Stipulations:

If you have any questions, please do not hesitate to contact Kay Powell in the IRB Office at 212-617-7525.

Good luck on your project.

cc: Michael Balick Ph.D.
     Biology

Sign the Verification Statement below. Return the original signed copy of this memo to the IRB Office and retain a copy for your records. The IRB Office must receive a copy of the signed verification statement before research may begin.

VERIFICATION:

By signing below, I acknowledge that I have received this approval and am aware of, and agree to abide by, all of its stipulations in order to maintain active approval status, including timely submission of continuing review applications and proposed protocol modifications, as well as prompt reporting of adverse events, serious unanticipated problems, and protocol deviations. I am aware that it is my responsibility to be knowledgeable of all federal, state and university regulations regarding human subjects research including CUNY’s Federalwide Assurance (FWA) with the Department of Health and Human Services Office of Human Research Protections.

Signature of Principal Investigator: ____________________________ Date: April 12, 2011

Signature of Faculty Advisor for Student Research: ____________________________ Date: April 5, 2011
Appendix C.2

Lehman College IRB Determination: Use of peripheral blood mononuclear cells (PBMCs) from healthy donor blood from the New York Blood Center determined "Not Human Subjects" research. Donors are deidentified.

DATE: December 16, 2013
TO: Daniel Kulakowski
FROM: Herbert H. Lehman College (CUNY) HRPP Office
PROJECT TITLE: [548091-1] Immunostimulant activity of a Micronesian tea
SUBMISSION TYPE: HUMAN SUBJECTS RESEARCH DETERMINATION
ACTION: DETERMINATION OF NOT RESEARCH
DECISION DATE: December 16, 2013

Thank you for your submission of the Human Subjects Research Determination Form for this project. It has been determined that this project does not meet the definition of human subject research as defined by the federal regulations (45 CFR 46.102(d) (?)) and therefore no further IRB review or approval is required. A human subject means a living individual about whom an investigator (whether professional or student) conducting research obtains (1) data through intervention or interaction with the individual, or (2) identifiable private information. The determination was made your study was not human subjects research because you will be utilizing peripheral blood mononuclear cells (PBMCs) that are obtained from de-identified blood samples provided by the New York Blood Center through their collaboration with Memorial Sloan-Kettering Cancer Center (MSKCC).

We will retain a copy of this correspondence within our records. If the scope of this project changes, you must return to the Herbert H. Lehman College (CUNY) HRPP Office for a determination of whether the project continues to not meet the definitions of human subjects research.

If you have any questions, please contact Tara Prairie at (718) 960-8960 or tara.prairie@lehman.cuny.edu. Please include your project title and reference number in all correspondence with this committee.

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within the City University of New York's records.
Appendix C.3

TCO Ref 09B5012-MTA

MATERIALS TRANSFER and Collaborative Research Agreement

This agreement ("Agreement"), effective as of December 31, 2008, is by and between Research Foundation of The City University of New York (hereinafter, "RFCUNY"), with principal offices located at 230 W. 41st Street, New York, NY 10036, on behalf of the City University of New York and Mr. Christopher Kitalong.

Whereas, Mr. Christopher Kitalong, a doctoral student of, City University of New York, is to bring plant specimens previously collected from the country of the Republic of Palau (hereinafter "ROP") and independently analyzed at Toyama Medical University in Japan into the laboratories of Professor Edward Kennelly at Lehman College of the City University of College for academic purposes;

Whereas, Mr. Kitalong shall conduct research project (defined below) involving said plant specimens in the laboratories of Dr. Kennelly;

Whereas, Dr. Kennelly agrees to instruct and collaborate with Mr. Kitalong to perform the research project as part of Mr. Kitalong's graduate education program;

Nowtherefore, the parties, in consideration of the mutual promises contained herein,

AGREE AS FOLLOWS:

1. Research Project
1.1 The parties will perform research related to phytochemical and biochemical-related studies (hereinafter, the "Research Project") of the Material (defined hereafter).

1.2 Lehman College of the City University of New York shall provide supplies for the Research Project.

2. The material (hereinafter "Material") that is covered by this Agreement includes the plant specimens and species listed in Exhibit A that are: a) indigenous to ROP, and b) have been released for research through Palau Bureau of Agriculture Quarantine under research permit no. BOA04-05, registered to Christopher Kitalong.

3. RFCUNY agrees that the Material is to be used solely for teaching and academic research purposes.

4. Mr. Kitalong represents that the Material has been collected in a manner consistent with the policies of the convention on Biological Diversity, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and local laws of the Republic of Palau relating to access and benefit sharing including, but not limited to, those related to traditional knowledge.

5. "Research Results" include any results, products or processes newly derived from the use of the Material in CUNY facilities during the course of the Research Project. Inventorship of any invention identified in the Research Results shall be determined in accordance with U.S. patent law and other commonly recognized patent policy in association with natural resources (if patentable), or by mutual agreement between the parties (if not patentable). Ownership shall follow inventorship. If it is determined that either Mr. Kitalong or Dr. Kennelly is an inventor of any invention identified in the Research Results, the Intellectual Property Policy of the City
University of New York shall apply, and RFCUNY will, as appropriate and in consultation with ROP, seek patent protection on such invention in US and/or abroad.

6. The parties recognize the need to compensate the ROP organizations and peoples in the event of commercialization of any invention identified in Research Results. In the event that RFCUNY desires to pursue commercialization of any such invention for which RFCUNY is a sole or joint owner, RFCUNY agrees to enter into good faith negotiation with ROP, represented by the appropriate department or agency at ROP, in a separate agreement which shall include terms regarding distribution of benefits, including but not limited to financial benefit. Such agreement shall be subject to any pre-existing obligations of the parties directly involved, including the Republic of Palau.

7. The parties agree to keep the Research Results confidential until approved for publication by all parties. If any party desires to submit a paper or abstract containing Research Results for publication, the other parties shall have up to 45 days prior to submission to review and, as necessary file a sole or joint patent application in accordance with section 5. The provisions of this Agreement shall not prevent or restrict any party’s right to publish the Research Results or use the Research Results for non-commercial research and educational purposes.

8. The parties agree to use the Material in a safe manner and in compliance with all laws, governmental regulations and guidelines applicable to the Material, including the current National Institutes of Health guidelines.

IN WITNESS WHEREOF, the parties hereto have caused this Agreement to be executed by their duly authorized representatives.

RFCUNY
By: ______________________
Name: Jake Maslow
Title: Director
Technology Commercialization Office
Date: 11/26/09

By: ______________________
Christopher Kitalong
Date: 2/09/2009
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<tr>
<th>Family</th>
<th>Species</th>
<th>Palauan name</th>
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<td>Verbenaceae</td>
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<td>ukali la ked</td>
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Exhibit A. List of Plant Species and Specimens from ROP
Appendix C.4
Rider to MTA Docket #09B5012, allowing Daniel Kulakowski to work under the previous MTA between The City University of New York and Christopher Kitalong.

**RIDER TO CUNY DOCKET #: TCO-09B5012-MTA**

"Material Transfer and Collaborative Research Agreement" between The City University of New York (herinafter, "CUNY") and Mr. Christopher Kitalong with reference to Intellectual Property Rights for plant materials collected in Palau and used for scholarly (e.g. non commercial) activity as part of the doctoral dissertation research program of Christopher Kitalong.

Whereas, Mr. Dan Kulakowski, a doctoral student at CUNY be working on plant material covered under the above mentioned CUNY Docket, previously collected from the Republic of Palau and independently analyzed at Toyama Medical University in Japan by Christopher Kitalong. Mr. Kulakowski will be analyzing the plant material in the laboratories of Professor Edward Kennelly at Lehman College of CUNY for academic purposes.

Mr. Kulakowski agrees to all stipulations in CUNY DOCKET #: TCO-09B5012-MTA and agrees in good faith that the research pursued is for educational and scholarly purposes and that the therapeutic uses of these plants have been established through the prior art and traditional knowledge and experimentation of the Palauan people.

Dan Kulakowski  
Doctoral Candidate  
The Graduate Center, CUNY  
December 28, 2010  
Date

Christopher Kitalong  
Doctoral Candidate  
The Graduate Center, CUNY  
The Environment, INC  
Koror, Palau 96940  
December 18, 2010  
Date
Appendix D

Peripheral Blood Mononuclear Cell (PBMC) culture protocol adapted from MSKCC Immune Monitoring Facility standard operating procedures

1. Ensure all vials, tubes, solutions, plates, solvent reservoirs, pipet tips are autoclaved or sealed sterile AND pyrogen-free
   a. Perform all work in biosafety cabinet, keeping airflow on

2. Prepare extract to required concentration (400-40 μg/ml ideal for crude extracts)
   a. This concentration will be 4x your concentration in treatment well
   b. If not soluble in 100% media, first resuspend in small amount of DMSO before adding media
      i. ensure final concentration of solvent DMSO in well no more than 0.5% of total volume
   c. Sonicate to ensure homogenous solution
   d. Sterile filter treatment

3. Thaw human PBMCs as quickly possible in 10% PHS/RPMI media
   a. fill 10 mL sterile centrifuge tube with 37 °C media
   b. thaw cells by placing in 37 °C water bath while flicking gently with your wrist
      i. when only a small chunk of ice remains in vial move on to next step
   c. add 1 mL of warmed media dropwise into vial containing frozen cells
   d. add media-frozen cell mixture to centrifuge tube containing media
   e. count cells
   f. dilute to 5x10^6 cells/ml (=0.5x10^6 cells/100 ul) in media

4. Put 100 ul of diluted PBMC in each testing well (half-million cells total/well)

5. Add 50 ul of diluted, resuspended plant extract

6. Add 50 ul of media OR immune stimulant (LPS, Melan-A, CEF if co-testing)
   a. Final concentration of botanical should be 10-100 μg/ml

7. Mix well and incubate at 37 °C for 72 hours
   a. ensure your incubator is humidified by adding a vessel of 0.5L sterile water to bottom of incubator
   b. this step is necessary to prevent evaporation of media

8. Collect 100 ul supernatant from each well for ELISA assay
   a. Generate supernatant by centrifuging plate at 1500 rpm x 5 mins
      i. Ensure no cells are collected with supernatant
b. If not immediately analyzing, store supernatant in individual vials at -80 °C

9. Use *Staphylococcus* enterotoxin B (SEB) as positive control

10. Run at least n=2 per treatment for significance
Appendix E:

**ELISA protocol for quantification of cytokines in cell-free supernatant**

*adapted from R&D Biosystems Quantikine ELISA protocol*

NOTE: This assay will take 2 days. 16 wells are needed for cytokine calibration curve.

1. Bring wash buffer, assay diluent, calibrator diluent and IFNγ conjugate to room temperature before use.

2. Dilute wash buffer
   a. 20 ml in 500 ml DI water, or 1:25 ratio if not all wells are being used.

3. Prepare IFNγ standard calibration curve
   a. If not reconstituted, refer to vial label for reconstitution volume.
   b. Stock solution concentration will be 1000 pg/ml.
   c. *Allow standard to sit for at least 15 minutes with gentle agitation prior to making dilutions.*
   d. Use stock solution (1000 pg/ml) to make serial dilutions (1000-15.6 pg/ml).
   e. Pipette 500 ul of calibrator diluent into 7 polypropylene tubes.
   f. Pipette 500 ul of stock solution into first tube, and continue with a dilution series.
      i. Mix each tube thoroughly before the next transfer.
      ii. Calibrator diluent serves as the zero standard.
      iii. total of 8 concentrations
          1. 1000, 500, 250, 125, 62.5, 31.2, 15.6, 0 pg/ml
   g. Any additional stock solution can be pipette into polypropylene tubes in 1 ml aliquots and frozen at -20C.

4. Remove excess microplate strips from the plate frame and return to foil pouch and reseal.

5. Add 100 ul of assay diluent to each well.

6. Add 100 ul of standard, **supernatant** or control to each well.
   a. Make sure addition is uninterrupted and completed within 15 minutes.
   b. Cover with adhesive strip and incubate for 2 hours at room temperature.

7. Aspirate each well and wash.
   a. Remove all reagents from well and discard.
   b. Fill each well with 400 ul wash buffer and remove.
   c. Repeated the process for a total of 4 washes.
   d. Remove any remaining wash buffer by decanting over a paper towel and aspirating with a pipet.
e. Cover remaining wash buffer with foil and keep in hood overnight

8. Add 200 ul of IFNγ conjugate to each well and cover with a new adhesive strip

9. Incubate overnight at room temperature (this step deviates from R&D Biosystems protocol). Promotes more repeatable results.

10. Bring color reagents A and B, and stop solution, to room temperature

11. Repeat aspiration/wash as in step 7

12. Prepare substrate solution from color reagents A & B
   a. Mix color reagents A & B together in equal volumes within 15 minutes of use and protect from light
   b. 200 ul of substrate solution is required per well

13. Add 200 ul of substrate solution to each well, cover with foil and protect from light
    a. Incubate for 30 minutes at room temperature

14. Add 50 ul of stop solution to each well. The color should change from blue to yellow.
    a. Mix well with pipet upon addition

15. Within 30 minutes determine the optical density of the wells on the spectrophotometer
    a. Record at two wavelengths, 450 and 540 (or 570 nm)
    b. Subtract readings at 540 or 570 nm from the readings at 450 nm to correct for optical imperfections in the plate

CALCULATIONS:
1. Average the replicate readings for each standard, control and sample and subtract the average optical density of the 0 pg/mL standard calibrator

2. Create a standard curve by plotting the mean absorbance for each standard concentration on the y-axis against the concentration on the x-axis and draw a best-fit line through the points on the graph
   a. Draw the best-fit line by regression analysis

3. Insert absorbance of unknown samples into calibration curve and solve for pg/mL cytokine
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