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Merging bioactivity with liquid chromatography-mass spectrometry-based chemometrics to identify minor immunomodulatory compounds from a Micronesian adaptogen, *Phaleria nisidai*

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ABSTRACT

This study presents a strategy based on repeatable reversed-phase LC-TOF-MS methods and chemometric statistical tools, including untargeted PCA and supervised OPLS-DA models, to identify low-yielding compounds with potent immunostimulant activity in *Phaleria nisidai* (Thymelaeaceae), a plant with a history of use as an adaptogen on the islands of Palau in Micronesia. IFNγ ELISA assays were used to classify chromatographic fractions according to imunomodulatory activity prior to LC-TOF-MS chemometric analysis to target and identify compounds likely to contribute to observed activity. Simplexin, a daphnane diterpene ester, was identified for the first time from this genus and caused an increase in the production of cytokines (IFNγ, IL1β, IL6, and IL13) by peripheral blood mononuclear cells. Five other daphnane diterpene esters were tentatively identified for the first time from this plant based on mass spectral data and are marker metabolites distinguishing active from inactive fractions. This analytical approach increased the efficiency of bioactivity-guided fractionation and has the potential to minimize redundant isolation and identify minor constituents with potent activity from a complex matrix.

1. Introduction

Liquid chromatography, paired with time-of-flight (TOF) and fragmentation (MS/MS) mass spectrometers, has enabled highly sensitive and efficient characterization of plant metabolic profiles [1,2]. Multivariate statistical analyses allow rapid examination of LC-MS metabolomic data from plant extracts [3,4]. When these techniques are combined with bioactivity analysis, the metabolites contributing medicinal activity to a plant can be efficiently determined.

Conventional methods of active compound discovery from natural products involve bioactivity-guided isolation. While this process is proved to be effective [5], a significant issue with this approach is the need for dereplication of compounds that are either well known or have non-specific effects [6]. It is difficult to characterize unknown individual components of a matrix until they are close to purity. If the active constituents are present in low abundance it becomes necessary to separate a large amount of material, which can be laborious, costly, or impossible to source. In addition, purification may remove unexpected minor constituents with potent activity.

The effects of underlying constituents with potent activity present at very low, and sometimes undetectable, levels can be misattributed to compounds present in greater abundance that are more readily detected. For example, triterpenes were thought to be responsible for anti-HIV activity in *Maprounea africana*, but as triterpene purity increased, the observed activity decreased, and it was determined that low-yielding daphnane-type diterpenes, undetectable by 500 MHz NMR, were responsible for the perceived activity [7]. The anti-tuberculosis activity of ursolic acid commercial standards was also seen to decrease as purity increased, implying that ursolic acid had synergistic effects with its impurities that led to observed antimycobacterial activity [8]. Anti-tuberculosis activity of *Oplopanax horridus* also decreased with increased purity of triterpenes, as purification removed small amounts of active monoglycerides [9].

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Phaleria nisidai Kaneh. is a small tree in the Thymelaeaceae family native to Palau, where it is also known as *delalakar*, translating to 'the Mother of medicine.' Ethnomedicinal interviews conducted with Palauan healers indicate that this plant is used as a system cleaner, to prevent and treat common sickness and flu, as an immune booster, and to enhance energy and strength. These traditional uses align with the indications of a tonic or panacea, a medicine that exerts healing influences throughout the body. The pharmacological term for a treatment with these effects is adaptogen; increasing "resistance to a very broad spectrum of harmful factors ('stressors') of different physical, chemical, and biological natures" [10].

A popular adaptogen [11] used in the United States is ginseng, one of the top 10 best-selling herbal dietary supplements in 2012 [12]. Many adaptogenic herbs act as immunomodulators, including Asian (*Panax ginseng*) and Siberian (*Eleutherococcus senticosus*) ginseng [11] from Traditional Chinese medicine, and ashwagandha (*Withania somnifera*) [13] and tulsi (*Ocimum sanctum*) [14] from Indian Ayurvedic medicine. Botanical immunomodulators can be employed in the treatment, prevention, or symptom-mitigation of cancer [15,16] and have also been utilized as adjuvants to increase immune system response to tumor vaccine [17,18] in cancer immunotherapy.

In this study, the immunomodulatory activity of various *P. nisidai* fractions on peripheral blood mononuclear cells (PBMCs) is recorded and orthogonal partial least-squares discriminatory analysis (OPLS-DA) is used to compare fractions at different bioactivity levels to infer the compounds responsible for adaptogenic use of this plant. Simplexin, the compound predicted by this analysis to have activity, was then screened in the original bioassay to confirm the utility of this approach to active compound identification from a complex matrix. This is also the first report of diterpene esters from the genus *Phaleria.*

A similar approach has been used by other groups to identify adenosine A1 receptor-binding compounds from different *Boesenbergia rotunda* extracts [19], thrombin-induced phosphorylation inhibitors from 43 green tea cultivars [20], and TNFα-inhibitory compounds from grapes and other berries [21]. 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.

2. Experimental

2.1. Chemical

LC-MS-grade MeCN, water, and formic acid were purchased from J.T. Baker (Philipsburg, NJ, USA) and GR grade MeOH, hexane, EtOAc, and isopropanol from VWR, Inc. (Bridgeport, PA, USA). Ultrapure water was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., Bedford, MA, USA). NMR solvents were bought from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Simplexin was provided by Dr. Mary Fletcher (Queensland Primary Industries and Fisheries, Animal Research Institute, Australia) and further confirmed by high-resolution MS, tandem MS, and ^{1}H NMR spectra.

2.2. Plant material

Samples of *P. 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.

2.3. Extraction

Bulk *P. nisidai* leaves collected as above were placed in an oven heated by a 30 W incandescent bulb until dry (approximately 72 h) and shipped to New York, where the leaves were powdered in a plant mill. 400 g of leaf powder was extracted in methanol under ultrasonic conditions for 2 h at room temperature (3 × 4 L). Methanol extracts were combined and concentrated under reduced pressure, yielding 76 g for bioactivity testing, metabolite analysis, and fractionation.

2.4. Pharmacological activity analysis

2.4.1. PBMC isolation

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, Buckinghamshire, UK) followed by washing with 10% fetal calf serum in phosphate-buffered saline (10% FCS/PBS). Cells were counted using an EMD Millipore Guava cell analyzer, diluted to appropriate concentration and frozen at −80 °C (Revco, Ashville, NC) 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, Carlsbad, CA) and brought up to 5×10^6 cells/mL for cell culture experiments.

2.4.2. Extract preparation

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.

2.4.3. Endotoxin testing

Following preparation in media, extracts, fractions, and isolated compounds were screened using the Endosafe Limulus amebocyte lysate (LAL) automated assay (Charles River Labs, Charleston, SC) with cartridges sensitive to 0.1 EU endotoxin/mL.

2.4.4. PBMC culture

One half-million cells (0.5×10^6 cells in 100 μL) 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 2.4.2. 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% CO_2). For ELISA experiments (Fig. 1), PBMCs from one healthy donor were used and cultured in triplicate. For multiarray cytokine analysis (Figs. 4 and 5), PBMCs from three healthy donors were used, and PBMCs from each donor were cultured in duplicate.

2.4.5. Cytokine analysis

Following incubation, cells were centrifuged and cell-free supernatant was harvested. A colorimetric ELISA kit (R&D Systems, Minneapolis, MN) was used for initial bioactivity-guided fractionation to measure IFNγ concentration in supernatant. One modification was made to manufacturer protocol. After adding