Traditional preparation of *Phaleria nisidai,* a Palauan tea, reduces exposure to toxic daphnane-type diterpene esters while maintaining immunomodulatory activity

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simplexin (PubChem CID: 119045); acetoxyhuratoxin (PubChem CID: 44559853); huratoxin (PubChem CID: 6375646)

ABSTRACT

Ethnopharmacological relevance: The leaves of *Phaleria nisidai* Kaneh. (Thymelaeaceae) are brewed into a tea commonly used as a tonic, strengthening beverage and immune enhancer in Palau, Micronesia. Recently, the leaves of *P. nisidai* have been shown to contain toxic daphnane diterpene esters which may pose a public health threat to Palauans.
Aims of the study: This project documents the use frequency, preparation and side effects of *Phaleria nisidai*. The content of daphnane diterpene esters in aqueous and methanol extracts and infusions prepared by healers in Palau is compared to assess the risk of daphnane ingestion associated with traditional consumption. Quantitative results are correlated with an *in vitro* assessment of the immunomodulating activity of the extracts.
Materials and methods: Research participants, comprising traditional healers and laypeople, were interviewed concerning use patterns and side effects of *Phaleria nisidai*. Several traditional healers prepared and provided boiled tea samples for chemical analysis. Leaves were collected and methanolic and aqueous extractions were prepared in the laboratory. Peripheral blood mononuclear cells (PBMCs) were cultured with various concentrations of methanol and aqueous leaf extracts and their output of IFNγ was measured using ELISA. Cell proliferation was also assessed using the MTT assay. The concentration of selected daphnane diterpene esters in healer-prepared infusions, lab methanol and lab aqueous extracts was quantified using ultraperformance liquid chromatography-mass spectrometry-triple quadrupole detection (UPLC-MS-TQD).

Results: Through structured interviews it was determined that *Phaleria nisidai* tea was used frequently, with many participants drinking it daily. The reported side effects were mild, and with the exception of diarrhea (n=2), no side effect was mentioned more than once. Methanol extracts contained 4.0 ug simplexin, 17.6 ug acetoxyhuratoxin and 2.3 ug huratoxin per g dry leaf material. In traditional water infusions provided by healers and in standardized lab-prepared aqueous extracts all three compounds were below the limit of detection (16.3 ng/mL) using our UPLC-MS-TQD method. Methanol and aqueous extracts increased the release of IFNγ by PBMCs (p<0.05); however, methanol extracts were significantly more active than aqueous extracts (p<0.05). Methanol and aqueous extracts significantly increased proliferation of PBMCs, causing at least 60% more cell proliferation than negative control (p<0.05).
Conclusions: The presence of daphnane diterpene esters in a frequently consumed traditional beverage was initially viewed as a public health concern, though interview data reveal that Palauans do not observe toxicity or side effects associated with their use of *Phaleria nisidai* tea. Concurrently, daphnanes are present in methanolic extracts but not detected in aqueous preparations indicating that the traditional method of preparation avoids the extraction of these potentially toxic compounds, while still maintaining immunostimulant activity.

1. Introduction

Phaleria nisidai Kaneh. (Thymelaeaceae) is a shrub to small tree native to the Republic of Palau, the most biodiverse country in Micronesia (Hinchley et al., 2007). It is known locally as *delal a kar*, which translates to "the mother of medicine" and has been referred to as a panacea by Palauans. As a component of broad, multi-year ethnobotanical studies by our group and collaborating institutions, including the Belau National Museum and the Pacific Academic Institute for Research (Dahmer et al., 2012; Graz et al., 2015), targeted interviews carried out for this work reveal that *P. nisidai* is one of the most widely-used plants in Palau and is employed

non-specifically to treat a variety of general health concerns. Its use as a prophylactic to prevent sickness, as a 'system cleaner', as well as for strength and energy indicate that it is being employed as an adaptogen, a medicine taken routinely to help adapt to stress caused by pathogenic, mental or physical sources (Brekhman and Dardymov, 1969). This plant is found throughout Palau, including wild populations growing on the limestone Rock Islands and sandy atolls, but is most frequently collected from home gardens or neighborhood trees on the most populated islands of Babeldaob and Koror.

2. Background

2.1. Bioactivity and chemistry

Previous pharmacological studies of *Phaleria nisidai* have demonstrated immunostimulatory activity in hydroalcoholic leaf extracts *in vivo* and *in vitro* (Matsuda et al., 2005, 2004). We have previously used the production of IFNγ by peripheral blood mononuclear cells (PBMCs) to guide fractionation of a methanol extract of *P. nisidai* and found that daphnane-type diterpene orthoesters contribute to the immunostimulatory activity of this plant (Kulakowski et al., 2014), which was the first report of diterpene esters from the *Phaleria* genus. These compounds possess interesting bioactivities, including anticancer (Liao et al., 2009) and immune modulation (Nakayasu et al., 1982); however an obstacle towards their further pharmaceutical development is that many daphnane diterpene esters are tumor promoters (Blumberg, 1988) and acute gastrointestinal irritants that have caused death in grazing animals (Fletcher et al., 2014; Freeman et al., 1979; Roberts et al., 1975).

Mangiferin (Matsuda et al., 2004), the benzophenone iriflophenone-2-*O*-α-rhamnoside and the flavonoid genkwanin-5-*O*-β-D-primeveroside (Kitalong et al., 2012), tetracosonol and a mixture of acylglucosylsterols (Matsuda et al., 2005) are the only compounds other than

daphnanes that have been identified from this plant. Mangiferin, the most abundant compound in aqueous extracts (Kitalong et al., 2012b), did not show immune stimulation in work done by Matsuda in *in vivo* and *in vitro* cytokine assays (Matsuda et al., 2004) and in our preliminary studies.

2.2. Ethnobotany of Phaleria nisidai

Based on ethnobotanical data obtained from various healers in Palau as part of ongoing studies at the Belau National Museum, as well as research undertaken for the *Palau Primary Healthcare Manual* (Dahmer et al., 2012), and studies on its phytochemistry (Kitalong et al., 2012a, 2012b) and clinical effectiveness (Graz et al., 2015), *Phaleria nisidai* was identified as one of the most widely-used medicinal plants in the country. In hundreds of use reports that our group and related researchers have collected *P. nisidai* is drunk as an aqueous infusion (Fig. 1), used as an aromatherapeutic in steam baths, or the whole leaf is applied topically, as a remedy or prophylactic for a number of diseases and ailments.

As part of this work, Palauans were interviewed to determine the use frequency, dose and side effects of this traditional medicine. Leaves of *Phaleria nisidai* were also collected from home gardens and wild populations for chemical and bioactivity analysis.

2.3. Collaborative ethnobotanical research and intellectual property rights issues

This work was performed under a collaborative research agreement between the City University of New York (CUNY) and the first and second authors of this work (material transfer agreement docket # TCO-09B5012-MTA). This document states that *Phaleria nisidai* is indigenous to Palau, has been released with the permission of the Palau Bureau of Agriculture, is to be used solely for academic research purposes, has been collected consistent with the policies set forth by the Convention on Biological Diversity, the Convention on International Trade in

Endangered Species and Wild Fauna and Flora (CITES), and local laws of the Republic of Palau. If any patentable or non-patentable inventions arise from this research it is agreed that organizations and peoples of the Republic of Palau will be compensated through a separate benefit-sharing agreement; including, but not limited to, financial benefit.

Informed consent was obtained before each interview; ensuring the research participant had adequate knowledge of what to expect as part of this research, that their intellectual property rights would be protected and that any economic benefits arising from this research would be shared between the Republic of Palau and its people. This research protocol was reviewed and approved by the CUNY institutional review board (IRB# 11-01-019-0135, granted to Daniel Kulakowski) and by the Palau Oral History Office.

2.4. Objective

The purpose of this work is to evaluate the bioactivity of *Phaleria nisidai* in assays relevant to its traditional medicinal preparation and use and determine the yields of three potentially toxic daphnanes; simplexin, huratoxin and acetoxyhuratoxin (Fig. 2) using ultra performance liquid chromatography-mass spectrometry-triple quadrupole detection (UPLC-MS-TQD). The amount of these compounds in laboratory methanol and water extracts and traditional aqueous teas made by Palauan healers was compared. This work will determine if the consumption of *P. nisidai* puts Palauans at risk of exposure to toxic compounds and relate these findings to any side effects and toxicity reported by research participants.

3. Experimental

3.1. Interviews

Research participants were identified as users of *Phaleria nisidai* through snowball, or chain-referral, sampling (Biernacki and Waldorf, 1981), based on the recommendations of

multiple personnel involved in this, and previous, studies. Participants provided informed consent in Palauan and English prior to the interview. Semi-structured interviews based on a questionnaire were often conducted in English, but Palauan was used depending on the preference of the participant with a field guide, Van-Ray Tadao, translating. A total of 26 traditional healers and laypeople were interviewed for this study.

3.2 Plant material

Samples of *Phaleria nisidai* were collected throughout Palau from March to July 2012. Leaves from a total of 229 trees were sampled and georeferenced, and for this study a subset of 27 trees were randomly selected by blind sampling, from the above collection. Local expert and botanist Ann Hillman Kitalong (Belau National Museum) identified the specimens. Voucher specimens were prepared and deposited at the Belau National Museum, Natural History Department (Collections DK001-DK034). Leaves were placed in a wood oven heated by four 30W incandescent light bulbs (~32 °C) until dry and stored at room temperature prior to shipment and extraction.

3.3. Chemical

LC-MS-grade MeCN, water and formic acid were purchased from J.T. Baker (Philipsburg, NJ, USA) and GR grade MeOH 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 purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Simplexin was provided by Dr. Mary Fletcher (Queensland Primary Industries and Fishers, Animal Research Institute, Australia) and further confirmed by high resolution MS, tandem MS and ^{1}H NMR spectra.

3.4. Extraction

One dry leaf from each of the 27 *Phaleria nisidai* trees (sec. 3.2) were combined and ground to powder in a spice mill. Aliquots of ground sample (50 mg) 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 min. HPLC-grade water (25 mL) was added to the remaining three samples, which were boiled in a sandbath at 100 °C for 30 min. All samples were then centrifuged for 7 min at 1760 × *g* (IEC Centra GP8, . The extract (5 mL) was decanted into vials, leaving the pellet behind. Methanolic extracts were dried under N_2 and aqueous extracts were frozen at -80 °C and lyophilized until dry.

Traditional extracts were prepared by six local healers in Palau. Healers collected seven fresh leaves from trees near their homes and boiled them in one gallon of water on the stove for 30 min. Individual infusions were decanted into plastic water bottles and frozen for shipment to our lab in New York City on dry ice in polystyrene coolers. 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 0.45 μm filter. Dried laboratory water extracts and traditional water infusions were resuspended in MS-grade H_2O to 5 mg/mL and passed through a 0.45 μm filter.

3.5. UPLC-MS-TQD Analysis

3.5.1. Separation and Detection

LC separations were conducted on an Acquity UPLC module (Waters Corp., Milford MA) using 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: 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.

Peaks were monitored, integrated and processed using Apex Tracking and the QuanLynx application of MassLynx software (Waters Corp.) with a retention time window of ±0.035-0.045 min.

3.5.2. Calibration curves

Simplexin was isolated by colleagues from *Pimelea elongata*, and used as a chromatographic standard. The purity of simplexin was determined to be 75.3%, based on the AUC of peaks in LC-MS-time of flight total ion chromatogram (positive mode) of the provided standard. 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. Due to lack of analytical standard, acetoxyhuratoxin and huratoxin were quantified according to simplexin equivalents (Baldini et al., 2014; Reynertson et al., 2008). Simplexin and huratoxin were quantified using the lower calibration curve (81.6, 163.3, 326.6 & 408.1 ng/mL injection concentrations) and

acetoxyhuratoxin was quantified using the upper calibration curve (816.3, 1632 & 2040 ng/mL injection concentrations) of injected simplexin standard, corrected for % purity.

The limit of detection (LOD) was determined chromatographically as the lowest injected concentration of simplexin (16.3 ng/mL) to give a repeatable (n=4) signal-to-noise ratio of at least 3:1. The limit of quantification (LOQ) was set as the lowest injected concentration to provide relative uncertainty (RSD) of <20%, determined to be 81.6 ng/mL.

3.6. Pharmacological activity analysis

3.6.1. Sample preparation for bioactivity testing

Methanol and lab-prepared water extracts of *Phaleria nisidai* were prepared for cell culture experiments. Samples were resuspended in DMSO, and then diluted to proper concentration with 10% human serum in RPMI 1640 media (10% HS/RPMI) (Gibco) so the final concentration of DMSO in each well was no more than 0.5% v/v.

3.6.2. Endotoxin screening

Once prepared for PMBC culture *Phaleria nisidai* extracts were screened using the Endosafe Limulus amebocyte lysate (LAL) automated assay (Charles River Labs, Charles River, SC) with cartridges sensitive to 0.1 EU endotoxin/mL.

3.6.3. Peripheral Blood Mononuclear Cell (PBMC) preparation

Buffy coat enriched in leukocytes was obtained from healthy de-identified donors from the New York Blood Center (Long Island City, NY). PMBCs were isolated by separation over Ficoll-PaquePLUS (GE Healthcare) followed by washing with 10% fetal calf serum in phosphate-buffered saline (Gibco). PBMCs were counted using a Guava easyCyte benchtop flow cytometer (EMD Millipore), diluted to appropriate concentration with freezing buffer and frozen

at -80 °C for 3 days, followed by long-term storage in liquid nitrogen. Upon use, cells were thawed in 10% HS/RPMI and diluted with media to $5x10^6$ cells/ml for cell culture.

3.6.4. Cell culture protocol

PBMCs ($0.5x10^6$ in 150 uL 10% HS/RPMI) were added to individual wells in a 96-well plate (400 µL/well). The cells were then treated with 50 uL methanol or water extracts of *Phaleria nisidai* for a final concentration of 62.5, 125 and 250 µg extract/mL media. Positive control consisted of PBMCs treated with 0.01 µg/ml *Staphylococcus* enterotoxin B (SEB) and negative control contained only media with 0.5% DMSO (v/v). All treatments were evaluated in triplicate culture experiments. Cells were incubated for up 72 h at 37 °C (5% CO_2). Cell culture conditions were similar for IFNγ determination and MTT cellular proliferation assay.

3.6.5. IFNγ evaluation

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

3.6.6. MTT protocol

To measure cell proliferation an MTT assay kit (Mosmann, 1983) was used (Roche Applied Science, Indianapolis, IN). After incubation 10 µL of MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid in phosphate buffered saline) was added to each well, and cells were incubated for another 4 h at 37 °C (5% CO_2). Solubilization solution (10% sodium dodecyl sulfate [SDS] in 0.01 M HCl) (100 µL) was added to each well and the

absorbance was measured at 590 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA) after overnight incubation.

3.7. 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 and standard errors.

4. Results

4.1. Ethnobotanical results

Data from interviews on use frequency, dose and side effects related to *Phaleria nisidai* tea are presented in Table 1. Everyday consumption was reported by twelve participants, three used it "almost every day" and four used it "whenever needed". Conversely, two participants used *P. nisidai* sporadically, mentioning that unspecific complications could result from frequent use.

No side effects were reported by eleven participants regarding the use of *Phaleria nisidai* in non-pregnant individuals; however, five of these participants mentioned that it should not be used by pregnant women. The remaining participants reported mild side effects, with only diarrhea being reported by more than one participant (n=2). One participant reported death can occur if too much of this infusion is consumed. While alarming, further details were not provided and it may be valuable to collect more information from this participant.

4.2. Bioactivity

4.2.1. Cytokine response

The production of IFNγ by PBMCs treated with methanolic and water extracts of *Phaleria nisidai* was measured using colorimetric ELISA techniques (Fig. 3). At doses of 62.5, 125 and 250 μg/ml the methanol extract exhibited immunostimulation, significantly greater than untreated cells. The results suggest 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 dose had intermediate response levels (1432.5 pg/mL) to the higher and lower doses, but was not significantly different from either. The methanol extract showed significantly higher activity than aqueous extracts at each concentration tested.

The aqueous *Phaleria nisidai* extract showed significant immunostimulation compared to negative control as well. At 62.5 and 125 μg/mL doses a two-fold increase in IFNγ production was observed (317.5 and 308.5 pg/mL respectively) over untreated cells (143.1 pg/mL) and the highest dose (250 μg/mL) caused more than a three-fold increase (502.7 pg/mL).

4.2.2. Cell proliferation

Water and methanol extracts both increased cell proliferation above negative control (Fig 4). Results are shown as % proliferation above untreated cells in the MTT assay. At 62.5 μg/mL the methanol extract (134.5%) caused significantly more proliferation than the water extract (88.2%) towards PBMCs, but at higher concentrations both extracts increased PBMC proliferation similarly. The methanol extract hints at an inverse dose-response but the decrease in proliferation was not significant.

4.3. Daphnane quantification by UPLC-MS-TQD

Simplexin was identified in methanol extracts of *Phaleria nisidai* based on MS and MS/MS transitions as well as retention time comparison to a chromatographic standard in UPLC-MS-TQD analysis. Simplexin eluted at a retention time of 2.36 min and relative retention

times and the transitions seen in Table 2 were used to identify two additional daphnanes; huratoxin and acetoxyhuratoxin, in methanol extracts. We have previously identified these compounds in *P. nisidai* fractions by LC-MS-ToF exact mass analysis (Kulakowski et al., 2014).

Simplexin, huratoxin and acetoxyhuratoxin were not detected (Table 3) 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. Average yields of three daphnanes from all extracts are listed in Table 3.

5. Discussion

Simplexin has been shown to have anticancer (Pettit et al., 1983), anti-HIV (Asada et al., 2011) and immunomodulatory activities (Nakayasu et al., 1982); however it also possesses irritant and tumor-promoting activities (Zayed et al., 1982). Similarly, acetoxyhuratoxin and huratoxin have anticancer activity (Abe et al., 1998; Weijan, 1992) contrasted with irritant and tumor-promoting activity (Adolf and Hecker, 1984, 1975; Adolf et al., 1988). Simplexin, acetoxyhuratoxin and huratoxin are known to be acutely toxic, and sometimes deadly, to livestock by damaging the gastrointestinal tract and causing heart failure (Fletcher et al., 2009; Roberts et al., 1975; Wilson et al., 2007). Previous work by our group revealed that these, and other daphnane compounds were present in hydrophobic *Phaleria nisidai* fractions with immunomodulatory activity (Kulakowski et al., 2014).

Interview data concerning side effects and toxicity associated with *Phaleria nisidai* use was examined due to the potential for harm related to daphnane exposure. This data revealed that users of this plant are not experiencing adverse health risks and have no empirical reason to be concerned with potential toxicity. It is used frequently (sometimes daily) and the only side effect mentioned more than once was diarrhea (two responses). All other side effects were mild, with

only one response each. The only grave threat mentioned came from one informant stating that drinking too much can kill a person. More details about this warning should be collected from this informant. The frequency of use and lack of caution associated with the consumption of this plant medicine by most informants led to the hypothesis that although daphnanes have been found in methanolic extracts of *P. nisidai* they are not likely to be present in acutely toxic amounts in traditional aqueous preparations.

Simplexin, acetoxyhuratoxin and huratoxin were detected and quantified in methanolic extracts of *Phaleria nisidai*, however these compounds were below the limit of detection (LOD = 16.3 ng/mL) in five laboratory-prepared water extracts and six aqueous infusions prepared by healers in Palau. Our interview data and results from previous researchers (Dahmer et al., 2012) indicate that fresh leaves are always boiled in water to make a tea, or that leaves are used in topical steam baths or aromatherapy. The strict use of aqueous preparations limits the exposure of *P. nisidai* users to these toxins. Preliminary studies suggest *P. nisidai* aqueous extracts have hepatoprotective activity (Kitalong et al., 2007).

The possibility remains that daphnanes are present in the aqueous tea in low amounts, undetectable using our LC-MS-TQD method. In a recent study cattle were fed with low daily doses of *Pimelea trichostachya* and simplexin was not detected with LC-MS/MS in tissue collected after autopsy. In addition, prolonged low-dose exposure reduced the toxic effects of this simplexin-containing plant. The authors hypothesize that the animals developed detoxification mechanisms for simplexin (Fletcher et al., 2014). A similar mechanism may be induced in Palauans who are being exposed to frequent low doses of daphnanes through the traditional aqueous preparation. A small clinical trial is underway that has shown no adverse

effects thus far, but a detailed pharmacokinetics study to understand the metabolism of these compounds would be important.

These results show that it is critical to follow the traditional preparation methods of botanicals with an ethnomedicinal history before conclusions can be drawn about toxicity and/or activity as it pertains to the cultural use of that plant. There are several cases of traditional, sometimes elaborate, preparation methods that exclude or eliminate toxins. For example, bark shavings of *Cinnamomum carolinense* are brewed in hot water as a medicine to treat back and joint pain in Pohnpei. This plant contains safrole, a known hepatotoxin, which is present in methanolic extracts. However, there has been 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) indicating that this plant is prepared in a way that maximizes its medicinal benefits while rendering its toxic components harmless. Similar cases have been shown with the traditional preparation of kava (*Piper methysticum*) (Currie and Clough, 2003; Moulds and Malani, 2003; Teschke et al., 2011) and cassava (*Manihot esculenta*) (Maduagwu and Oben, 2007; Okafor et al., 2002).

5. Conclusion

Aqueous extracts of *Phaleria nisidai* that mimic the traditional preparation of this culturally important Palauan medicinal plant were shown to have bioactivity relevant to its traditional use as an immunostimulant. The activity of aqueous extracts was reduced compared to methanolic extracts and this may be due to the higher concentration of daphnane diterpene esters in methanol extracts, which were undetectable in aqueous preparations. These compounds are responsible for toxicity and death in livestock and are potent tumor promoters in mouse models of carcinogenesis; however, they cannot be detected in traditionally prepared *P. nisidai*

infusions. The high use-frequency of this medicine along with scarce reporting of side effects or toxicity indicates that the traditional aqueous preparation reduces exposure to daphnanes in the user. The possibility remains that daphnanes are present in trace amounts in the aqueous preparations of *P. nisidai*, sufficient to confer an adaptogenic benefit, while avoiding acute toxicity. While the long-term toxicity of *P. nisidai* use has been not revealed in ethnobotanical data, it may be important to clinically investigate signs of chronic toxicity in this population.

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References

Abe, F., Iwase, Y., Yamauchi, T., Kinjo, K., Yaga, S., Ishii, M., Iwahana, M., 1998. Minor daphnane-type diterpenoids from *Wikstroemia retusa*. Phytochemistry 47, 833–837.

Adolf, W., Hecker, E., 1975. On the irritant and cocarcinogenic principles of *Hippomane mancinella*. Tetrahedron Letters 16, 1587–1590. doi:10.1016/S0040-4039(00)72205-2

Adolf, W., Hecker, E., 1984. On the active principles of the spurge family, X. Skin irritants, cocarcinogens, and cryptic cocarcinogens from the latex of the manchineel tree. Journal of natural products 47, 482–496.

Adolf, W., Seip, E., Hecker, E., 1988. Irritant principles of the mezereon family (Thymelaeaceae), V. New skin irritants and tumor promoters of the daphnane and 1α-alkyldaphnane type from *Synaptolepis kirkii* and *Synaptolepis retusa*. Journal of Natural Products 5, 662–674.

Asada, Y., Sukemori, A., Watanabe, T., Malla, K.J., Yoshikawa, T., Li, W., Koike, K., Chen, C.-H., Akiyama, T., Qian, K., Nakagawa-Goto, K., Morris-Natschke, S.L., Lee, K.-H., 2011. Stelleralides A-C, novel potent anti-HIV daphnane-type diterpenoids from *Stellera chamaejasme* L. Organic Letters 13, 2904–7.Baldini, M., Ferfuia, C., Bortolomeazzi, R., Verardo, G., Pascali, J., Piasentier, E., Franceschi, L., 2014. Determination of phorbol esters in seeds and leaves of *Jatropha curcas* and in animal tissue by high-performance liquid chromatography tandem mass spectrometry. Industrial Crops and Products 59, 268–276. Biernacki, P., Waldorf, D., 1981. Snowball sampling: problems and techniques of chain referral sampling. Sociological Methods Research 10, 141–163.

Blumberg, P.M., 1988. Protein kinase C as the receptor for the phorbol ester tumor promoters. Sixth Rhoads Memorial Award Lecture 1–8.

Brekhman, I.I., Dardymov, I. V, 1969. New substances of plant origin which increase nonspecific resistance. Annual review of pharmacology 9, 419–30.

Currie, B.J., Clough, A.R., 2003. Kava hepatotoxicity with Western herbal products: does it occur with traditional kava use? The Medical Journal of Australia 178, 421–2.

Dahmer, D.S.M., Balick, D.M.J., Kitalong, A.H., Kitalong, C., Herrera, K., Law, D.W., Lee, D.R., Tadao, V.-R., Rehuher, F., Hanser, S., Soaladaob, K., Ngirchobong, G., Besebes, M., Wasisang, F., Kulakowski, D., Adam, I., 2012. Palau Primary Health Care Manual: Health Care in Palau: Combining Conventional Treatments and Traditional Uses of Plants for Health and Healing. The New York Botanical Garden, Ministry of Health, Republic of Palau, The Continuum Center for Health and Healing and Belau National Museum. Charleston SC.

Fletcher, M.T., Chow, S., Ossedryver, S.M., 2014. Eff ect of increasing low-dose simplexin exposure in cattle consuming *Pimelea trichostachya*. Journal of Agricultural and Food Chemistry 62, 7402-06.

Fletcher, M.T., Silcock, R., Ossedryver, S., Milson, J., Chow, S., 2009. Understanding Pimelea Poisoning of Cattle. Department of Employment, Economic Development and Innovation, Queensland. 44 p.

Freeman, P., Ritchie, E., Taylor, W., 1979. The constituents of Australian *Pimelea* spp. I. The isolation and structure of the toxin of *Pimelea simplex* and *P. trichostachya* Form B responsible for St. George disease of cattle. Australian Journal of Chemistry 32, 2495.

Graz, B., Kitalong, C., Yano, V. 2015. Traditional local medicine in the republic of Palau and non-communicable diseases (NCD), signs of effectivenss. Journal of Ethnopharmacology 161, 233-7.

Hinchley, D., Lipsett-Moore, G., Sheppard, S., Sengebau, F.U., Verheij, E., Austin, S., 2007. Biodiversity Planning for Palau's Protected Areas Network An Ecoregional Assessment. TNC Pacific Island Countries Report No. 1/07. The Natural Conservancy.

Hojsgaard, S., Halekoh, U., 2013. doBy - Groupwise summary statistics, general linear contrasts, population means (least-squares-means), and other utilities.

Kitalong, C., Lee, S.S., Hattori, M. 2007. Hepatoprotective effects of Palauan medical plants. Poster presented at: The 24th Annual Meeting of Medical and Pharmaceutical Society for WAKAN-YAKU. Sept 8-9. Toyama, Japan.

Kitalong, C., Ali, M., El-Dine, R., 2012a. Phenolics from *Phaleria nisidai* with estrogenic activity. Records of Natural Products 3, 296–300.

Kitalong, C., Tadao, V., Hillmann, A., Balick, M., Kennelly, E., 2012b. Phytochemical analysis of Palauan traditional medicinal plant *Phaleria nisidai* to determine therapeutic dosing of mangiferin, a C-glucosyl xanthone. Planta Medica 78, PF1.

Kulakowski, D.M., Wu, S.-B., Balick, M.J., Kennelly, E.J., 2014. Merging bioactivity with liquid chromatography-mass spectrometry-based chemometrics to identify minor immunomodulatory compounds from a Micronesian adaptogen, *Phaleria nisidai*. Journal of Chromatography A 1364, 74–82.

Liao, S.-G., Chen, H.-D., Yue, J.-M., 2009. Plant orthoesters. Chemical Reviews 109, 1092–140.

Maduagwu, E.N., Oben, D.H.E., 2007. Effects of processing of grated cassava roots by the "screw press" and by traditional fermentation methods on the cyanide content of gari. International Journal of Food Science & Technology 16, 299–302.

Matsuda, H., Tokunaga, M., Hirata, N., Iwahashi, H., Naruto, S., Kubo, M., 2004. Studies on Palauan medicinal herbs. I.: Antidiabetic effect of ongael, leaves of *Phaleria cumingii* (Meisn.) F. Vill. Natural Medicines 58, 278–283.

Matsuda, H., Tokunaga, M., Iwahashi, H., Naruto, S., Yagi, H., Masuko, T., Kubo, M., 2005. Studies on palauan medicinal herbs. II. Activation of mouse macrophages RAW 264.7 by ongael, leaves of *Phaleria cumingii* (Meisn.) F. Vill. and its acylglucosylsterols. Biological & Pharmaceutical Bulletin 28, 929–33.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Moulds, R.F.W., Malani, J., 2003. Kava: herbal panacea or liver poison? The Medical Journal of Australia 178, 451–3.

Nakayasu, M., Terada, M., Adolf, W., Opferkuch, H.J., Schmidt, R., 1982. Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. Journal of Cancer Research and Clinical Oncology 103, 17–29.

Okafor, P.N., Okorowkwo, C.O., Maduagwu, E.N., 2002. Occupational and dietary exposures of humans to cyanide poisoning from large-scale cassava processing and ingestion of cassava foods. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 40, 1001–5.

Pettit, G., Zou, J., Goswami, A., 1983. Antineoplastic agents, 88. *Pimelea prostrata*. Journal of Natural Products 46, 563–568.

R Core Team, 2013. R: A language and environment for statistical computing.

Revelle, W., 2013. psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, IL.

Reynertson, K. a, Balick, M.J., Lee, R., Raynor, W., Pelep, Y., Kennelly, E.J., 2005. A traditional method of *Cinnamomum carolinense* preparation eliminates safrole from a therapeutic Pohnpean tea. Journal of Ethnopharmacology 102, 269–74.

Reynertson, K. a, Yang, H., Jiang, B., Basile, M.J., Kennelly, E.J., 2008. Quantitative analysis of antiradical phenolic constituents from fourteen edible Myrtaceae fruits. Food Chemistry 109, 883–890.

Roberts, H.B., McClure, T.J., Ritchie, E., Taylor, W.C., Freeman, P.W., 1975. The isolation and structure of the toxin of *Pimelea simplex* responsible for St. George disease of cattle. Australian Veterinary Journal 51, 325–326. Teschke, R., Sarris, J., Glass, X., Schulze, J., 2011. Kava, the anxiolytic herb: back to basics to prevent liver injury? British Journal of Clinical Pharmacology 71, 445–8.

Teschke, R., Qiu, S.X., Tran, D.X., Lebot, V. 2011. Kava and kava hepatotoxicity: requirements for novel experimental, ethnobotanical and clinical studies on a review of the evidence. Phytotherapy Research 25, 1263-74.

Weijan, F., 1992. Studies on antitumour active compouds of *Stellera chamaejasme* L. and their mechanisms of action. Journal of the Medical Society of Toho University 38, 896.

Wilson, S.-J., Taylor, J.D., Gibson, J.A., McKenzie, R.A., 2007. *Pimelea trichostachya* poisoning (St. George disease) in horses. Australian Veterinary Journal 85, 201–5.

Zayed, S., Adolf, W., Hecker, E., 1982. On the active principles of the Thymelaeaceae. I. The irritants and cocarcinogens of *Pimelea prostrata*. Journal of Medicinal Plant Research 45, 67–77.

Figures Legends

Fig. 1. Typical preparation of a *Phaleria nisidai* aqueous infusion. Leaves are washed, placed in a pot and boiled in water for at least 15 mins over a stovetop or woodfire until a clear amber-brown liquid (the color of black tea) results. Photo by Van-Ray Tadao.

Fig. 2. Chemical structures of daphnane diterpene esters quantified in crude *Phaleria nisidai* extracts.

Fig. 3. Concentration of IFNγ (pg/mL) in PBMC culture as a result of treatment with *Phaleria nisidai* methanol and water extracts. Negative control (NC) was treatment with DMSO (0.5% v/v) and media. *Staphylococcus* enterotoxin B (SEB) was used as positive control at 0.01 μg/ml. Results expressed as mean ± standard error (n=3). Upper case letters denote significant differences among methanol extracts. Lower case letters denote significant differences among water extracts. Levels with the same letters are not significantly different from one another ($p<0.05$). All methanol and water doses are significantly different from negative control ($p<0.05$).

Fig. 4. PBMC proliferation after treatment with crude methanol and crude water extract at three doses. Values indicate % proliferation above negative control (0.5% DMSO in media). *Staphylococcus* enterotoxin B (SEB) at 0.01 μg/ml used as positive control. Results expressed as mean ± standard error ($n=3$). * indicates methanol and water extracts are significantly different from one another ($p<0.05$) for a given concentration. All extracts caused significantly greater proliferation than negative control.

Table 1. Side effects and frequency of use information from 26 research participants.

side effects and contraindications	responses	use frequency	responses
no side effects[a]	11	everyday	12
diarrhea	2	whenever needed	4
too much will decrease insulin	1	almost every day	3
do not drink if trying to get pregnant	1	2-3 times per week	2
weight loss	1	throughout the day for one day every other week	1
do not eat with fruits	1	7 days straight, 2-4 times a year	1
make women bleed more	1		
unspecific side effects if you drink too much	1		
mild dizziness with overdose	1		
death with overdose	1		

[a]No side effects reported for non-pregnant users. Of these 11 responses, 5 participants would not recommend using this medicine when pregnant.

Table 2. TQD-MS parameters used for quantification of daphnane diterpene orthoesters

compound	RT (min)	quantification SRM	confirmation SRM	LOD ng/mL	LOQ ng/mL	slope	inter cept	R^2
simplexin[a]	2.36	533.5 > 253.2	533.3 > 267.2	16.3	81.6	.4431	4.340	.999
huratoxin[a]	2.87	585.5 > 253.3	585.5 > 267.2			.4431	4.340	.999
acetoxyhuratoxin[b]	2.75	643.3 > 207.2	643.3 > 277.2			.3326	81.48	.999

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

[a]simplexin and huratoxin curve constructed from lower range of simplexin calibration curve (81.6 to 408.1 ng/ml, 4 points)

[b]acetoxyhuratoxin curve constructed from upper range of simplexin calibration curve (489.7 to 2041 ng/ml, 4 points)

Table 3. Concentration of daphnane diterpene orthoesters detected in *Phaleria nisidai* extracts

extract		simplexin (µg/g DW)	acetoxyhuratoxin (µg/g DW)[a]	huratoxin (µg/g DW)[a]
lab MeOH extracts		4.0 ± 0.6	17.6 ± 3.3	2.3 ± 0.4
lab H_2O extracts		n.d.[b]	n.d.	n.d.
traditional H_2O	TI	n.d.	n.d.	n.d.
	TB	n.d.	n.d.	n.d.

preparations	TT	n.d.	n.d.	n.d.
	Obak	n.d.	n.d.	n.d.
	TNgsar	n.d.	n.d.	n.d.
	Tnger	n.d.	n.d.	n.d.

All values are expressed as means ± standard deviation of quadruplicate injections of three extraction replicates.

[a]Expressed as simplexin equivalent (semi-quantitative).

[b]n.d., not detected; below the limit of detection.

Graphical Abstract

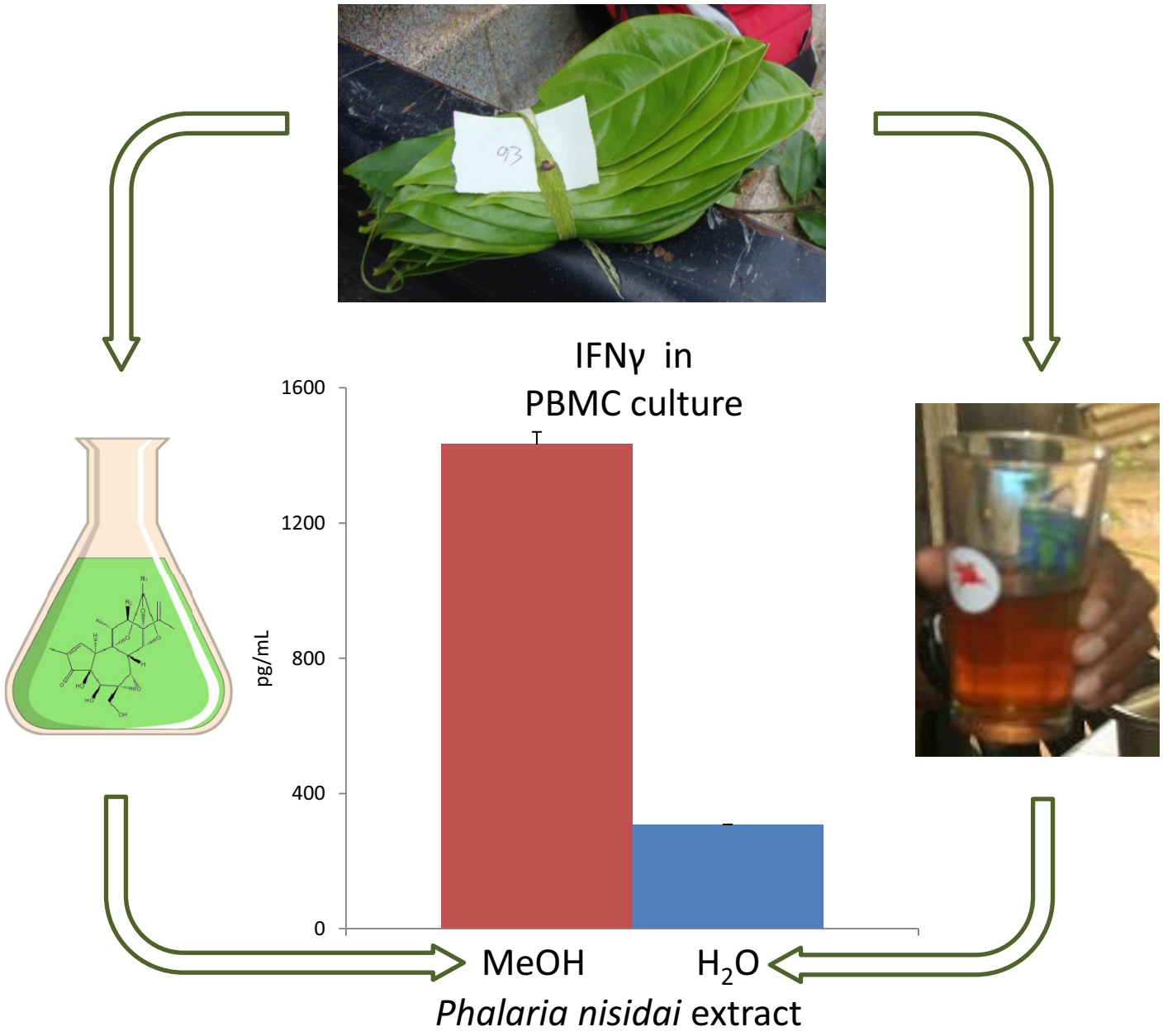
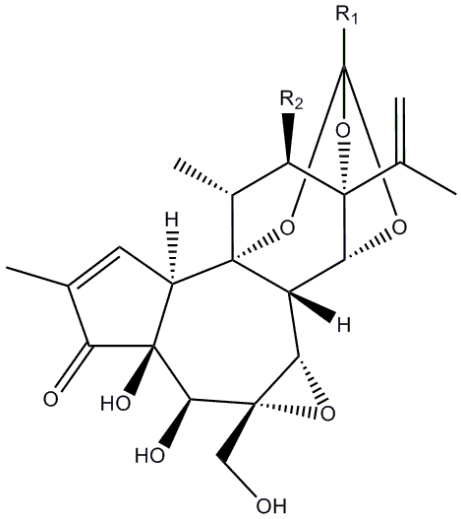


Figure 1

Figure 2



compound	R
simplexin	R_1: C_9H_{19} R_2: H
huratoxin	R_1: $(CH{=}CH)_2(CH2)_8CH_3$-*(E,E)* R_2: H
acetoxyhuratoxin	R_1: $(CH{=}CH)_2(CH2)_8CH_3$-*(E,E)* R_2: AcO

Fig. 1. Chemical structures of daphnanes quantified in crude *Phaleria nisidai* extracts

Figure 3

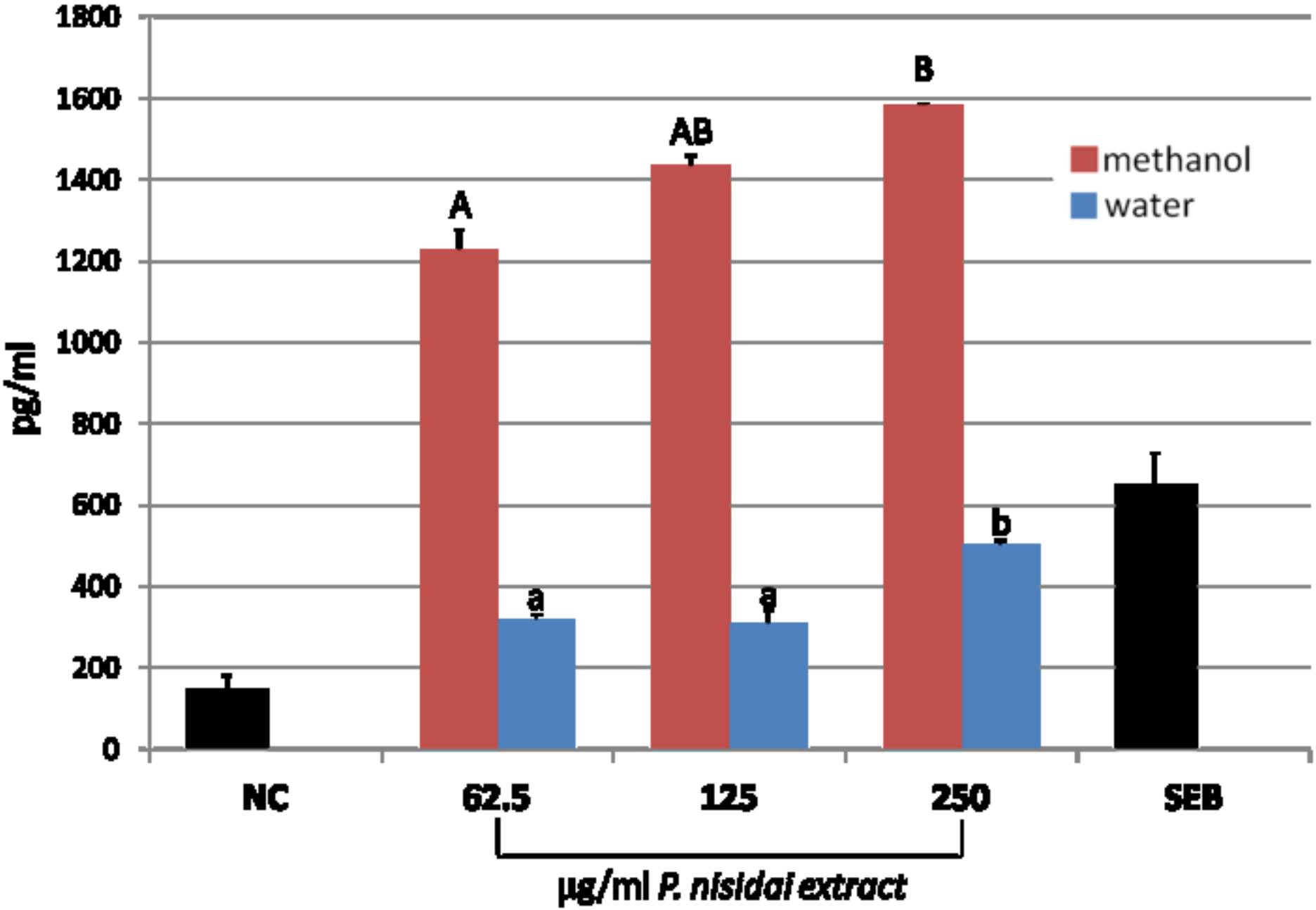
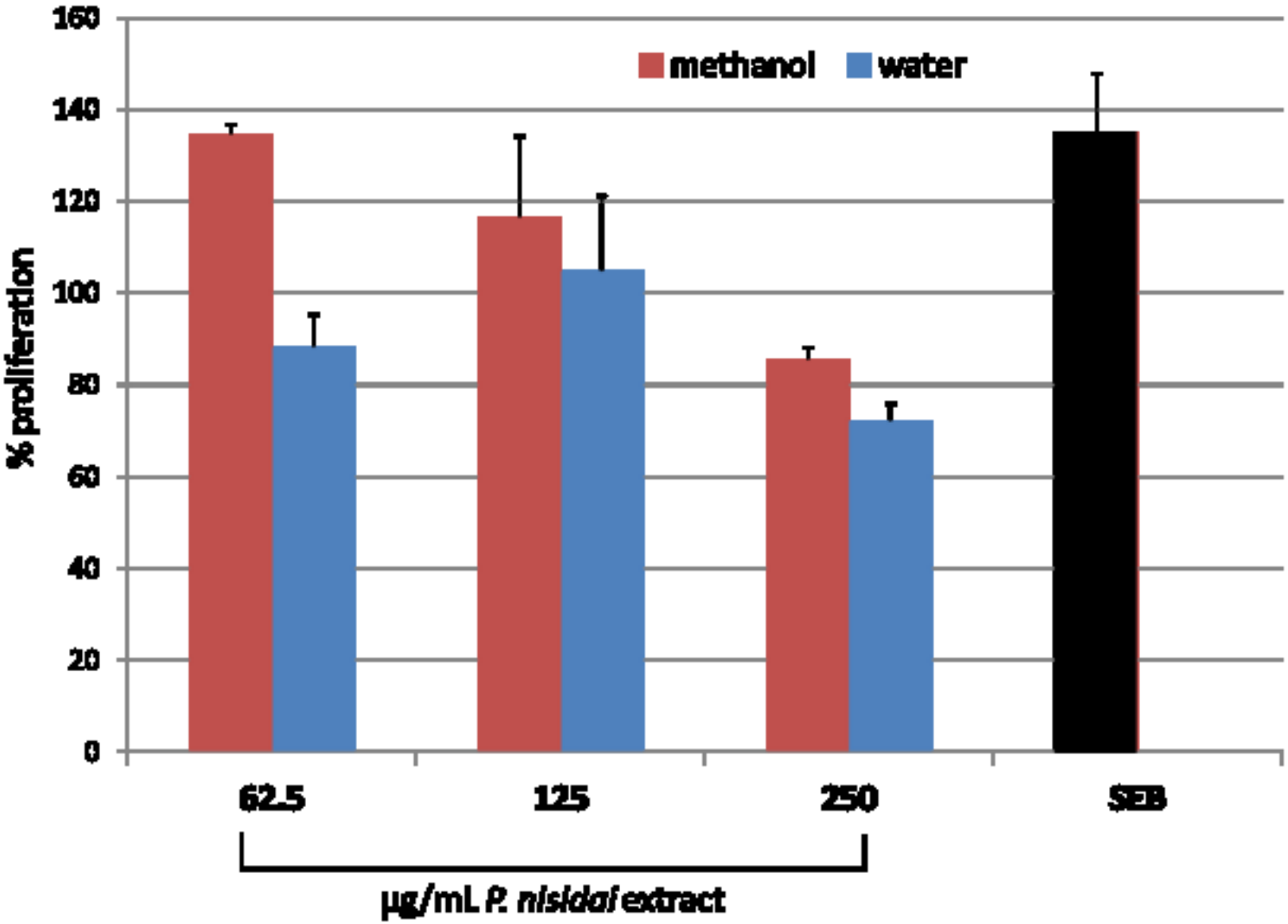

1800
1600
1400
1200
1000
800
600
400
200
0
pg/ml
A
AB
B
a
a
b
methanol
water
NC
62.5
125
250
SEB
µg/ml P. nisidai extract

Figure 4


160
140
120
100
80
60
40
20
0
% proliferation
methanol
water
62.5
125
250
SEB
µg/mL P. nisidai extract