Journal of
Medicinal Plant Research

Volume 11 Number 40, 25 October, 2017

ISSN 1996-0875

Academic Journals

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Journal of Medicinal Plants Research

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Vol. 11(39), pp. 621-634, 25 October, 2017
DOI: 10.5897/JMPR2017.6485
Article Number: 854FF7566460
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Revisiting the linkage between ethnomedical use and development of new medicines: A novel plant collection strategy towards the discovery of anticancer agents

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Received 31 August, 2017; Accepted 3 October, 2017

The Vietnam-Laos International Cooperative Biodiversity Group (ICBG) based at the University of Illinois at Chicago (UIC) catalyzed a country-wide network of medicinal plant preserves (MPP) and medicinal biodiversity preserves (MBP) now established in ten provinces of the Lao People's Democratic Republic (Lao PDR), which are relied upon as protected sources of ethnomedicines for local villagers and traditional healers. In collaboration with the Lao PDR's Institute of Traditional Medicine (ITM), our ongoing P01 Program Project (Ohio State University) examined the anticancer bioprospecting potential for two of the most exhaustively inventoried of these sites: the Bolikhamxay MPP and the Xiengkhouang MBP. Guided by prior voucher specimens sourced from these reserves, with an overwhelming emphasis on plants employed in traditional medicine, 201 distinct samples from 96 species were collected along with proper herbarium documentation. Aliquots of these plant samples were extracted in azeotropic ethanol and evaporated to dryness for initial biological evaluation. In six samples from six different species (2.99% of the collected samples and 6.25% of taxa), it was observed that extracts exhibited notable cytotoxicity against HT-29 colon adenocarcinoma cells. The wisdom behind the utilization of HT-29 cells in this preliminary biological screen is discussed. Furthermore, comparison of screening results based on longstanding considerations and ideological underpinnings of ethnobotanical vs. "random" biodiversity-based collection approaches is detailed herein. The results of this interdisciplinary study support the hypothesis that, by privileging the initial sample set in terms of human safety and pharmacological activity, ethnobotanically driven collection for biological screening efforts can produce leads unprecedented by the strict traditional usages of plants.

Key words: Lao People's Democratic Republic (PDR), medicinal plants, traditional medicine, cancer.

INTRODUCTION

In trusting human agency and the intentionality of traditional ecological knowledge, one may be liable to conclude that botanical ethnopharmacopeias consist of plants that: (a) bear useful pharmacological activities and

can improve health status and (b) can be administered to patients in ways that render them clinically safe within reasons (Fadeyi et al., 2013; Pan et al., 2013; Getasetegn and Teferi, 2016). Given these two likelihoods, these ethnopharmacopeias can be studied in their entirety through biological screening efforts (Mazzio and Soliman, 2009; Fadeyi et al., 2013; Leonti and Weckerle, 2015; Odonne et al., 2017), setting the stage in the drug discovery pipeline towards human health applications not necessarily anticipated by indigenous knowledge and folk clinical application of the particular plants evaluated in this way. It might even be expected that this strategy could yield results superior to "random" collection efforts constrained to geographic areas with similar levels of species richness and biodiversity (Bletter, 2007; Saslis-Lagoudakis et al., 2012). Prior meta-analysis suggests that on a per sample basis, depending on ethnobotanical use and screening assays performed for samples evaluated from Laos and Vietnam, plants employed in traditional medicine can have a higher hit rate for bioactivity in empirical studies (Gyllenhaal et al., 2012). Although there are caveats and nuances to this finding, which will be discussed subsequently, this insight is worth bearing in mind in the context of the present paper. The hypothesis of this study, accordingly, states that the agentive, purposive nature of botanical ethnopharmacopeias biases sample sets derived from them for useful bioactivity; this selection criterion therefore lends itself to success in initial biological screening and drug discovery studies.

Towards the end of further, and unambiguous exploration of the possibility for ethnobotanically driven screening efforts, in this instance for the preliminary stages of anticancer drug discovery, two of the most extensively inventoried preserves in the Lao PDR, the Xiengkhouang MBP and the Bolikhamxay MPP were selected as promising expedition sites for the extramurally funded Program Project (P01) from among the ten preserves that are extant (Sydara et al., 2014; Soejarto et al., 2015) (Figure 1). These reservoirs for local traditional medicine plants served as the premiere P01 project sites for the exploration of the ethnopharmacopeias of Laos through the lens of this pragmatic, serendipitous-activity-through-human-utility paradigm. This paper presents the results of this endeavor.

METHODOLOGY

Memorandum of agreement

A Memorandum of Agreement (MOA) for the conduct of collaborative research targeted to the flowering plants, between the University of Illinois at Chicago and the Institute of Traditional Medicine (ITM, Vientiane, Lao PDR), covering issues on intellectual property and the sharing of benefits in the event of the discovery and development of a pharmaceutical product was established. This MOA allowed for the collection of plant materials (plant samples and their voucher herbarium specimens) in Laos and their transfer to and biological evaluation in the USA.

Plant collection

Following the signing of this agreement, a joint plant collection expedition between the University of Illinois at Chicago (UIC) and the Institute of Traditional Medicine (ITM) was undertaken in the Lao PDR. One expedition site was the Xiengkhouang Medicinal Biodiversity Preserve (MBP) of the Kham District, Xiengkhouang Province, and the other was Bolikhamxay Medicinal Plant Preserve (MPP) of the Paksan District, Bolikhamxay Province (Figure 1).

Xiengkhouang medicinal biodiversity preserve expedition

The Xiengkhouang Medicinal Biodiversity Preserve (MBP) (as depicted in Figure 1) is located at about 50 km northeast of the capital city (Phonsavanh) of Xiengkhouang Province, near Ban Tha, a rural Lao Lum (Lowland Lao) village in the Kham District, and about 15 km from both Muang Kham and Ban Tha. The elevation is approximately 1,140 m above sea level, at 19°43' N; 103°35' E (GPS reading). This preserve comprises approximately 500 hectares of high quality, secondary, montane tropical rainforest (Figure 2).

Two of the ITM's 5-passenger Toyota Hilux pickup trucks, with collapsible soft tops for covering the rear cargo area, provided excellent mobility throughout the expedition period (December 8 to 13, 2015). These vehicles allowed for the transportation of 6 to 10 passengers (including drivers) in addition to the loads of collected plant materials and field supplies. Two workers and several other locals were employed from the village of Ban Tha for the purposes of harvesting and processing plant material.

Near the Xiengkhouang MBP, quarters at the Seng Deuane guest house in Meuang Kham were rented as the expedition base and as equipment and supplies storage. The concrete patio of the guest house served as the base for drying plant samples and working quarters. The space and facilities of this guest house permitted the efficient performance and completion of the expedition within a short time period. The warm and mostly dry conditions in the winter season during the expedition eased the initial stages of processing of the voucher specimens and the plant samples collected.

Part of the supplies and equipment for use in the expedition, such as tarpaulins, rice sacks, nylon mesh bags, cardboard, plant presses, strings, branch cutters, twig clippers, knives (pointed knives known locally as *mid* [/mi:d/]), a GPS, digital cameras, binoculars, used newspapers, and other plant collecting and processing supplies, was brought from Vientiane through the ITM's supplies or as part of the ITM and UIC personnel's belongings. Forays into the field and the search for the plants collected were guided by a list and photographs of plants previously collected (Soejarto et al., 2015) from this MBP. Plant samples and voucher herbarium specimens were collected, carefully numbered and documented by field notes and photographic images. Numbered herbarium specimens were pressed between newspapers in the

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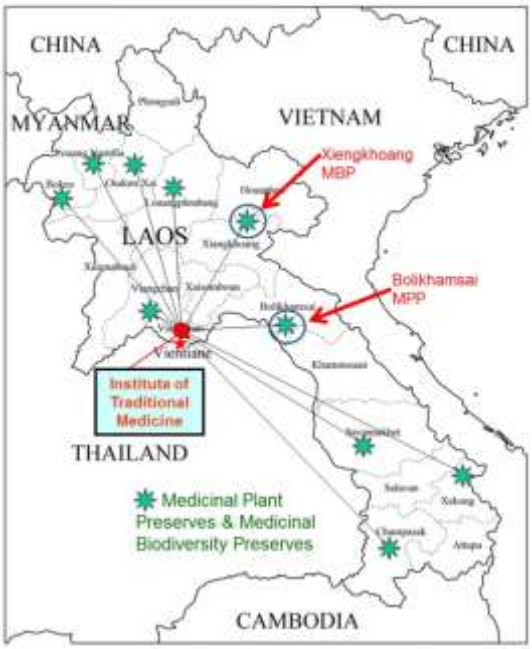

Towards the end of further, and unambiguously, exploring the possibility for ethnobotanically-driven screening efforts, in this instance for the preliminary stages of anticancer drug discovery, two of the most extensively inventoried preserves in the Lao PDR, the Xiengkhouang MBP and the Bolikhamxay MPP, were selected as promising expedition sites for our Program Project (P01) from among the ten preserves that are extant (Sydara et al., 2014; Soejarto et al., 2015) (Fig. 1). These reservoirs for local traditional medicine plants served as the premiere P01 project sites for the exploration of the ethnopharmacopeias of Laos through the lens of this pragmatic, serendipitous-activity-through-human-utility paradigm. This paper presents the results of this endeavor.

Figure 1. Medicinal plants preserve and medicinal biodiversity preserves network of Lao PDR (Soejarto et al., 2015), showing the location of expedition sites described in this paper.

field. Screening samples, 1 to 3 kg fresh weight, depending on nature or fleshiness of the plant material, were packed in sample collection bags made of nylon mesh and were placed in the trucks. On return from the field, these were arranged in rows to dry on the concrete patio of the Seng Deuane guest house, only being removed to the interior of the guest house or a nearby wooden, roofed platform when the conditions were overcast. At the end of the field operation (December 13, 2015), semi-dry samples in the nylon mesh bags were loaded into the trucks, while voucher herbarium specimens in newspapers were cinched in straps and cardboard sheets and also loaded into the trucks. All field equipment and plant materials collected were transported by the pickup trucks to the ITM, where the drying of the samples and voucher herbarium specimens was completed.

Bolikhamxay medicinal plant preserve expedition

The Bolikhamxay Medicinal Plant Preserve (MPP), also known as the Somsavath MPP due to its propinquity, usefulness and political connection to Somsavath Village, is about 27 km south of Pakxan, the capital city of Bolikhamxay Province and is 163 m above sea level, at 18°27' N; 103°48' E. The preserve comprises approximately 13 hectares of high quality, secondary, lowland broad-leaved tropical rainforests recovering from past fires and logging (Figure 3), with adjoining land cleared for agriculture and plantations, primarily, economic botanicals (*Hevea* rubber, agarwood, etc.).

One of the ITM's 5-passenger Toyota Hilux pickup trucks, with a collapsible soft top for covering the rear cargo area, provided excellent mobility throughout the expedition period (December 14-17, 2015). This vehicle allowed for the transportation of 3 to 5 passengers (including drivers) in addition to the loads of collected plant materials and field supplies. Guest house quarters in Pakxan were rented as the expedition base and as equipment and supplies storage. The concrete patio and blacktop of the guesthouse served as a base for drying plant samples and working quarters. The space and facilities of this guesthouse permitted the efficient performance and completion of the expedition within a short time period.

As in the Xiengkhouang expedition, part of the supplies and equipment for use in the expedition was brought from Vientiane through the ITM's supplies or as part of the ITM and UIC personnel's belongings. In the collection area, one worker and several other locals were employed from the village of Ban Khampai for the purposes of harvesting and processing plant material. Forays into the field and the search for plants to be collected were guided by a list and by photographs of the plants previously collected (Soejarto et al., 2015) from this MPP. The warm and mostly dry conditions during the winter season of the expedition eased the initial stages of processing for voucher specimens and screening samples in the field. The roofed cement floor of the visitor center, near where the automobiles were usually parked, served as a temporary processing and drying area before the plant materials were loaded into the cargo bay of the Toyota Hilux on returning to the Pakxan guesthouse.

Plant samples and voucher herbarium specimens were carefully numbered and documented by field notes and photographic images. Numbered herbarium specimens were pressed between newspapers in the field. Screening samples, 1 to 3 kg fresh weight, depending on nature or fleshiness of the plant material, were packed in sample collection bags made of nylon mesh and were placed in the truck during forays. Later these were arranged in rows on the concrete patio of the guesthouse. At the end of the field operation (December 17, 2015), semi-dry samples in the nylon mesh bags were loaded into the truck, while voucher herbarium specimens in newspapers were cinched in straps and cardboard sheets. All were transported by the pickup truck to the ITM, where the drying of the samples and voucher herbarium specimens was completed.

Figure 2. Xiengkhouang Medicinal Biodiversity Preserve, December 9-11, 2015. Top left: Roadside signage downslope of the expedition site. Top right: View of the montane tropical rainforest on a cloudy day with forbs and soil in the foreground. Bottom left: View of the tree cover from the roadside. Bottom right: Forested rivulet within the preserve.

Plant identification

One set of voucher herbarium specimens was shipped, processed and accessioned at the John G. Searle Herbarium of the Field Museum of Natural History (F), Chicago. Two additional sets of vouchers were deposited at the herbaria of the ITM. Taxonomic identification of the plants collected was initially performed (by JMH, KS, OS, MX, DDS) at the ITM Herbarium and was completed at the herbarium of the Field Museum.

Plant extraction

Azeotropic ethanolic extracts of the plant samples were generated from 30 g aliquots of plant samples at the laboratories of the ITM. Hence, 30 g aliquots of the 201 unground plant samples were milled and subsequently macerated twice overnight in 250 ml, followed by 200 ml of solvent, successively. The pooled extracts from each sample were desiccated by rotary evaporation at 40°C and transferred to small vials for shipment. All extracts were dispatched to and received safely at the University of Illinois at Chicago, where they were submitted to the MTS assay to determine their effect on the viability of HT-29 colon adenocarcinoma cells.

Colorimetric MTS assay for cell viability

Human colon cancer cells HT-29 were purchased from the American Type Culture Collection (Manassas, VA). The cell line was propagated at 37°C in 5% CO_2 in RPMI 1640 medium, supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells in log phase growth were harvested by trypsinization followed by two washings to remove all traces of the enzyme. A total of 5,000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96®, Falcon) and incubated overnight (37°C in 5% CO_2). Samples dissolved in DMSO were then diluted and added to the appropriate wells (concentrations: 20 and 2 µg/ml; total volume: 100 µl; DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37°C and evaluated for viability with a commercial absorbance assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corp Promega) measured viable cells. Survival percentage, based on microplate reader (Synergy Mx, BioTek) readings of absorbance at 490 nm, was expressed in percentage relative to the solvent (DMSO) control. One of the authors (Dr. Wei-Lun Chen) performed the bioassay and interpreted the results (Ren et al., 2017).

RESULTS

Fieldwork

A total of 201 plant samples for preliminary biological screening, comprising 96 species of seed plants,

Figure 3. Bolikhamxay Medicinal Plant Preserve, December 15-16, 2015. Top left: Roadside signage at the entrance of the preserve. Top right: View from the road of the path into the preserve, on left. Bottom left: Processing samples on the patio of the pavilion near the entrance. Bottom right: Lowland tropical rainforest within the preserve featuring trees, treelets, lianas, and forbs.

documented by 96 sets of voucher herbarium specimens, were gathered during the two expeditions (Table 1).

MTS assay

At least six samples out of the 201 collected presented with activity bearing enough interest for further testing with HT-29 colon adenocarcinoma cells, using the colorimetric MTS assay for cell viability, that is, cytotoxicity. At the 20 µg/ml incubation condition, less than 60% of the HT-29 cells in these samples survived, and in five out of six of these samples, less than 50% of the HT-29 cells survived, indicating that the IC_{50} of these five extracts should be under 20 µg/ml. Given that all six samples were from different taxa, this means that 2.99% of the collected samples and 6.25% of taxa with samples that were screened in this way were sufficiently cytotoxic to merit initial recollection for further studies.

The six samples of interest (Table 2 and Figure 4) are the following: the stem material of *Cryptolepis dubia* (Burm.f.) M.R.Almeida (A07194/ST; Asclepiadaceae); the aerial parts of *Rubia argyi* (H.Lév. & Vaniot) Hara ex Lauener (A07196/PX; Rubiaceae); the fruits of *Reevesia pubescens* Mast. (A07214/FR; Sterculiaceae); the combined leaves, twigs, and fruits of *Maclura tricuspidata* Carrière (A07257/LF+TW+FR; Moraceae); the stem material of *Millettia pachyloba* Drake (A07338/ST; Fabaceae-Papilionoideae); and the leaves and twigs of *Gardenia annamensis* Pit. (A07365/LF+TW; Rubiaceae).

DISCUSSION

Usage of HT-29 and other human tumor cell lines in anticancer screening of plants

The prevalence of cytotoxic taxa in this sample set

Figure 4. Plants from the Xiengkhouang Medicinal Biodiversity Preserve with samples exhibiting notable cytotoxicity in HT-29 colon adenocarcinoma cells. A: *Cryptolepis dubia*. B: *Rubia argyi*. C: *Reevesia pubescens*. D: *Maclura tricuspidata*. E*: Millettia pachyloba*. F: Gardenia annamensis.

derived from two expeditions in the Lao PDR is generally consistent with the results anticipated by the laboratory personnel of the P01CA125066 grant (Kinghorn et al., 2009, 2016). The HT-29 cell line was selected as the sole gatekeeper for initial cytotoxicity testing, from amongst a number of human tumor cell lines accessible to Ohio State University scientists involved with the program project. HT-29 was the only human tumor cell line on hand that displayed high selectivity for the most cytotoxic extracts through its low susceptibility, with unpublished, internal project data suggesting that a signature of only ~5% (or less) of sample extracts evaluated bore significant activity against HT-29 cells. In terms of the P01 project itself, the consequent focus on HT-29 cells and human colon adenocarcinoma by association has led to screening for inhibitory activity in the K-*ras* pathway, becoming one of the major mechanistic assays emphasized (Peruchot et al., 1987; Ren et al., 2014). HT-29 cells are also significant in that the cell line has long been and continues to be included in the NCI-60 panel utilized for the COMPARE algorithm to predict mechanism of action for cytotoxic compounds (Paull et al., 1989; Shoemaker, 2006). More broadly HT-29 cells are derived from a human colon adenocarcinoma, and this subtype of colon cancer, derived from the epithelial lining surrounding the lumen of the large intestine, is responsible for over 90% of colon cancer cases (Kumar et al., 2010). According to the most current data from the Center for Disease Control, furnished with the assistance of the National Cancer Institute, as of 2013 colorectal cancer is the third most prevalent cancer shared by both sexes in the United States of America and is the second deadliest (Center for Disease Control, 2014).

Aside from the P01 project's established reasoning for proceeding first with cytotoxicity screening using HT-29 cells under the aegis of the National Cancer Institute, the results of this initial P01 expedition and bioassay work in the Lao PDR also show reasonable consistency with prior bio-prospecting findings of the Vietnam-Laos ICBG project (1998-2012) (Gyllenhaal et al., 2012). It should be noted that the National Cooperative Drug Discovery Group ("Novel Strategies for the Discovery of Plant-Derived Anticancer Agents") (Kinghorn et al., 2003; Balunas et al., 2006), the intellectual predecessor of the P01CA125066 program project, overlaps substantially with the Vietnam-Laos ICBG (Kinghorn et al., 2003, 2009) in both time period (1990-2006) and the cell lines employed in cytotoxicity testing. Cell lines tested for viability against plant extracts during the ICBG included human colon cancer (COL-2), human promyelocytic leukemia (HL-60), human telomerase reverse transcriptase-retinal pigment epithelial cells (hTERT-RPE1), human umbilical vein endothelial cells (HUVEC), human cervical carcinoma, formerly believed to be oral carcinoma (KB); human prostate carcinoma (LNCaP), human lung cancer (LU-1), and human breast cancer

Table 1. Plant samples from the December 2015 P01 expeditions in the Lao PDR, listed in order of their voucher herbarium collection and by their scientific name (species-family), number and part of the plant of the primary screening samples, and locations.

Voucher herbarium specimen	Species (Family)[a]	Primary samples (Plant parts)	Collection location
JMH 001	*Medinilla septentrionalis* (W.W. Sm.) H.L. Li (Melastomataceae)	A07186/ST; A07187/RT; A07188/LF+TW+FL+FR	Xiengkhouang MBP
JMH 003	*Micromelum falcatum* (Lour.) Tanaka (Rutaceae)	A07189/RT; A07190/ST; A07191/LF+TW+FR	Xiengkhouang MBP
JMH 004	*Derris scandens* (Roxb.) Benth. (Fabaceae-Papilionoideae)	A07192/ST; A07193/LF+TW	Xiengkhouang MBP
JMH 005	*Cryptolepis dubia* (Burm.f.) M.R. Almeida (Asclepiadaceae)	A07194/ST; A07195/LF+TW	Xiengkhouang MBP
JMH 006	*Rubia argyi* (H.Lév. & Van.) Hara ex Lauener (Rubiaceae)	A07196/PX	Xiengkhouang MBP
JMH 008	*Actinodaphne rehderiana* (C.K. Allen) Kosterm. (Lauraceae)	A07197/ST; A07198/LF+TW	Xiengkhouang MBP
JMH 009	*Cissampelos pareira* L. (Menispermaceae)	A07199/PX	Xiengkhouang MBP
JMH 010	*Clematis leschenaultiana* DC. (Ranunculaceae)	A07200/PX	Xiengkhouang MBP
JMH 011	*Vitex quinata* (Lour.) F.N. Williams (Verbenaceae)	A07201/FR; A07202/LF+TW; A07203/ST	Xiengkhouang MBP
JMH 012	*Schefflera* cf. *leucantha* R. Vig. (Araliaceae)	A07204/FR+TW; A07205/LF+TW; A07206/ST	Xiengkhouang MBP
JMH 013	*Elsholtzia blanda* (Benth.) Benth. (Lamiaceae)	A07207/PX	Xiengkhouang MBP
JMH 014	*Saurauia napaulensis* DC. (Actinidiaceae)	A07208/FR+TW; A07209/LF+TW	Xiengkhouang MBP
JMH 016	*Clematis subumbellata* Kurz (Ranunculaceae)	A07210/PX	Xiengkhouang MBP
JMH 017	*Mucuna bracteata* DC. (Fabaceae-Papilionoideae)	A07211/PX	Xiengkhouang MBP
JMH 018	*Ilex* sp. (Aquifoliaceae)	A07212/ST; A07213/LF+TW+FR	Xiengkhouang MBP
JMH 021	*Reevesia pubescens* Mast. (Sterculiaceae)	A07214/FR; A07215/LF+TW; A07216/ST	Xiengkhouang MBP
JMH 022	*Schima* wallichii (DC.) Korth. (Theaceae)	A07217/LF+TW+FR; A07218/ST	Xiengkhouang MBP
JMH 023	*Rourea minor* (Gaertn.) Alston (Connaraceae)	A07219/ST; A07220/LF+TW; A07221/FR	Xiengkhouang MBP
JMH 024	*Acacia pennata* (L.) Willd. (Fabaceae-Mimosoideae)	A07222/FR; A07223/LF+TW; A07224/ST	Xiengkhouang MBP
JMH 025	*Wendlandia uvariifolia* Hance ssp. *laotica* (Pit.) Cowan (Rubiaceae)	A07225/FR; A07226/LF+TW	Xiengkhouang MBP
JMH 026	*Wikstroemia meyeniana* Warb. (Thymelaeaceae)	A07227/ST; A07228/RT; A07229/LF+TW+FR	Xiengkhouang MBP
JMH 027	*Engelhardia roxburghiana* Wall. (Juglandaceae)	A07230/SB; A07231/ LF+TW+FR; A07232/SW	Xiengkhouang MBP
JMH 028	*Rhus chinensis* Mill. (Anacardiaceae)	A07233/LF+TW+FR	Xiengkhouang MBP
JMH 029	*Rotheca serrata* (L.) Steane & Mabb. (Verbenaceae)	A07234/LF+TW+FL; A07235/ST	Xiengkhouang MBP
JMH 030	*Vernonia arborea* Buch.-Ham. (Asteraceae)	A07236/LF+FL; A07237/ST	Xiengkhouang MBP
JMH 031	*Uncaria sessilifructus* Roxb. (Rubiaceae)	A07238/LF+TW+FL; A07239/ST	Xiengkhouang MBP
JMH 032	*Debregeasia longifolia* (Burm.f.) Wedd. (Urticaceae)	A07240/LF+TW+FR; A07241/ST	Xiengkhouang MBP
JMH 036	(Lamiaceae?)	A07242/PX	Xiengkhouang MBP
JMH 037	*Casearia graveolens* Dalzell (Flacourtiaceae)	A07243/LF+TW+FR; A07244/ST	Xiengkhouang MBP
JMH 038	*Pittosporum napaulense* (DC.) Rehder & E.H. Wilson (Pittosporaceae)	A07245/LF+TW+FR; A07246/ST	Xiengkhouang MBP
JMH 039	*Eurya laotica* Gagnep. (Theaceae)	A07247/LF+TW+FR; A07248/ST	Xiengkhouang MBP
JMH 040	*Melastoma imbricatum* Wall. ex Triana (Melastomataceae)	A07249/LF+TW; A07250/ST	Xiengkhouang MBP
JMH 041	*Rubus alceifolius* Poir. (Rosaceae)	A07251/LF+TW; A07252/ST	Xiengkhouang MBP
JMH 042	*Rubus pluribracteatus* L.T.Lu & Boufford (Rosaceae)	A07253/LF+TW; A07254/ST	Xiengkhouang MBP
JMH 043	*Ligustrum sinense* Lour. (Oleaceae)	A07255/LF+TW; A07256/ST	Xiengkhouang MBP

Table 1. Cont'd.

JMH 044	*Maclura tricuspidata* Carrière (Moraceae)	A07257/LF+TW+FR; A07258/ST	Xiengkhouang MBP
JMH 045	*Elephantopus mollis* Kunth (Asteraceae)	A07259/PL	Xiengkhouang MBP
JMH 046	*Tadehagi triquetrum* (L.) H. Ohashi (Fabaceae-Papilionoideae)	A07260/PL	Xiengkhouang MBP
JMH 048	*Itea macrophylla* Wall. (Iteaceae)	A07261/LF+TW+FR; A07262/SB; A07263/SW	Xiengkhouang MBP
JMH 049	*Rhynchotechum ellipticum* (Wall. ex D. Dietr.) A. DC. (Gesneriaceae)	A07264/LF+FR; A07265/ST	Xiengkhouang MBP
JMH 052	*Symplocos lancifolia* Sieb. & Zucc. (Symplocaceae)	A07266/LF+TW; A07267/SB; A07268/SW	Xiengkhouang MBP
JMH 053	*Chloranthus spicatus* (Thunb.) Makino (Chloranthaceae)	A07269/PX	Xiengkhouang MBP
JMH 055	*Buddleja asiatica* Lour. (Buddlejaceae)	A07270/LF+TW+FL; A07271/ST	Xiengkhouang MBP
JMH 057	*Cayratia tenuifolia* (Wight & Arn.) Gagnep. (Vitaceae)	A07272/LF+TW+FR; A07273/ST	Xiengkhouang MBP
JMH 058	*Oreocnide integrifolia* (Gaudich.) Miq. (Urticaceae)	A07274/LF+TW+FR; A07275/ST	Xiengkhouang MBP
JMH 059	*Gynura divaricata* (L.) DC. (Asteraceae)	A07276/PX	Xiengkhouang MBP
JMH 060	*Deeringia amaranthoides* (Lam.) Merr. (Rosaceae)	A07277/PX	Xiengkhouang MBP
JMH 061	*Anneslea fragrans* Wall. (Theaceae)	A07278/LF+TW; A07279/SB	Xiengkhouang MBP
JMH 062	*Blumea* sp. (Asteraceae)	A07280/PX	Xiengkhouang MBP
JMH 063	*Tithonia diversifolia* (Hemsl.) A.Gray (Asteraceae)	A07281/LF+TW+FL; A07282/ST	Xiengkhouang MBP
JMH 065	*Engelhardia spicata* Lesch. ex Bl. (Juglandaceae)	A07283/LF+TW; A07284/SB; A07285/SW	Xiengkhouang MBP
JMH 068	*Omphalea bracteata* (Blanco) Merr. (Euphorbiaceae)	A07286/ST; A07287/LF+TW	Bolikhamxay MPP
JMH 069	*Baccaurea ramiflora* Lour. (Euphorbiaceae)	A07288/LF+TW; A07289/ST	Bolikhamxay MPP
JMH 070	*Vitex stylosa* Dop (Verbenaceae)	A07290/LF+TW; A07291/ST	Bolikhamxay MPP
JMH 071	*Ancistrocladus tectorius* (Lour.) Merr. (Ancistrocladaceae)	A07292/LF+TW; A07293/ST	Bolikhamxay MPP
JMH 072	*Lasianthus trichophlebus* Hemsl. ex F.B. Forbes & Hemsl. (Rubiaceae)	A07294/LF+TW; A07295/ST	Bolikhamxay MPP
JMH 073	*Psychotria cephalophora* Merr. (Apocynaceae)	A07296/LF+TW; A07297/ST	Bolikhamxay MPP
JMH 074	*Dracaena cambodiana* Pierre ex Gagnep. (Agavaceae)	A07298/LF+TW; A07299/ST	Bolikhamxay MPP
JMH 075	*Gardenia* cf. *annamensis* Pit. (Rubiaceae)	A07300/LF+TW; A07301/ST	Bolikhamxay MPP
JMH 076	*Eurycoma longifolia* Jack (Simaroubaceae)	A07302/LF+TW; A07303/ST	Bolikhamxay MPP
JMH 077	*Mussaenda glabra* Vahl (Rubiaceae)	A07304/LF+TW; A07305/ST	Bolikhamxay MPP
JMH 078	*Bauhinia penicilliloba* Pierre ex Gagnep. (Fabaceae-Caesalpinioideae)	A07306/LF+TW; A07307/ST	Bolikhamxay MPP
JMH 079	*Gnetum macrostachyum* Hook. f. (Gnetaceae)	A07308/PX	Bolikhamxay MPP
JMH 080	*Breynia fleuryi* Beille (Euphorbiaceae)	A07309/PX	Bolikhamxay MPP
JMH 081	*Garcinia celebica* L. (Clusiaceae)	A07310/LF+TW; A07311/ST	Bolikhamxay MPP
JMH 082	*Connarus paniculatus* Roxb. (Connaraceae)	A07312/LF+TW; A07313/ST	Bolikhamxay MPP
JMH 083	*Kibatalia laurifolia* (Ridl.) Woodson (Apocynaceae)	A07314/LF+TW; A07315/ST	Bolikhamxay MPP
JMH 084	*Cratoxylum formosum* (Jacq.) Benth. & Hook. f. ex Dyer (Clusiaceae)	A07316/ST	Bolikhamxay MPP
JMH 085	*Knema erratica* (Hook. f. & Thomson) J. Sinclair (Myristicaceae)	A07317/LF+TW; A07318/SB; A07319/SW	Bolikhamxay MPP
JMH 086	*Pterospermum argenteum* Tardieu (Sterculiaceae)	A07320/LF+TW; A07321/SB; A07322/SW	Bolikhamxay MPP
JMH 087	*Arenga caudata* (Lour.) H.E. Moore (Arecaceae)	A07323/PX	Bolikhamxay MPP
JMH 088	*Lithocarpus* cf. *toumorangensis* A. Camus (Fagaceae)	A07324/LF+TW; A07325/SB; A07326/SW	Bolikhamxay MPP

Table 1. Cont'd.

JMH 089	*Jasminum* cf. *annamense* Wernham ssp. *glabrescens* P.S.Green (Oleaceae)	A07327/PX	Bolikhamxay MPP
JMH 090	*Alpinia calcarata* (Haw.) Roscoe (Zingiberaceae)	A07328/LF; A07329/ST; A07330/RT	Bolikhamxay MPP
JMH 091	*Amomum* cf. *villosum* Lour. (Zingiberaceae)	A07331/LF; A07332/ST; A07333/RT	Bolikhamxay MPP
JMH 092	*Thysanolaena* cf. *latifolia* (Roxb. ex Hornem.) Honda (Poaceae)	A07334/LF; A07335/ST	Bolikhamxay MPP
JMH 093	*Cyperus trialatus* (Boeckeler) J. Kern (Cyperaceae)	A07336/PL	Bolikhamxay MPP
JMH 094	*Millettia pachyloba* Drake (Fabaceae-Papilionoideae)	A07337/LF+TW; A07338/ST	Bolikhamxay MPP
JMH 095	*Saccharum arundinaceum* Retz. (Poaceae)	A07339/PX	Bolikhamxay MPP
JMH 096	*Miscanthus* cf. *sinensis* Andersson (Poaceae)	A07340/LF; A07341/ST	Bolikhamxay MPP
JMH 097	*Lagerstroemia balansae* Koehne (Lythraceae)	A07342/LF+TW; A07343/SB; A07344/SW	Bolikhamxay MPP
JMH 098	*Barringtonia pauciflora* King (Lecythidaceaee)	A07345/LF+TW; A07346/ST	Bolikhamxay MPP
JMH 099	*Ormosia cambodiana* Gagnep. (Fabaceae-Papilionoideae)	A07347/LF+TW; A07348/SB; A07349/SW	Bolikhamxay MPP
JMH 100	*Aporosa ficifolia* Baill. (Euphorbiaceae)	A07350/LF+TW; A07351/ST	Bolikhamxay MPP
JMH 101	*Ardisia conspersa* E. Walker (Myrsinaceae)	A07352/LF+TW+FR; A07353/ST	Bolikhamxay MPP
JMH 102	*Securidaca inappendiculata* Hassk. (Polygalaceae)	A07354/LF+TW; A07355/ST	Bolikhamxay MPP
JMH 103	*Syzygium* cf. *chloranthum* (Duthie) Merr. & L.M.Perry (Myrtaceae)	A07356/LF+TW; A07357/ST	Bolikhamxay MPP
JMH 104	*Peltophorum dasyrrhachis* (Miq.) Kurz (Fabaceae-Caesalpinioideae)	A07358/LF+TW; A07359/SB; A07360/SW; A07361/RT	Bolikhamxay MPP
JMH 105	*Capparis trinervia* Hook. f. & Thomson (Capparidaceae)	A07362/ST	Bolikhamxay MPP
JMH 106	*Aporosa tetrapleura* Hance (Euphorbiaceae)	A07363/LF+TW; A07364/ST	Bolikhamxay MPP
JMH 107	*Gardenia annamensis* Pit. (Rubiaceae)	A07365/LF+TW; A07366/SB; A07367/SW	Bolikhamxay MPP
JMH 108	*Macaranga denticulata* (Bl.) Muell.-Arg. (Euphorbiaceae)	A07368/LF+TW; A07369/SB; A07370/SW	Bolikhamxay MPP
JMH 109	*Maesa ramentacea* (Roxb.) A. DC. (Myrsinaceae)	A07371/LF+TW; A07372/ST	Bolikhamxay MPP
JMH 111	*Sandoricum koetjape* (Burm.f.) Merr. (Meliaceae)	A07373/LF+TW; A07374/SB; A07375/SW	Bolikhamxay MPP
JMH 112	*Tetrameles nudiflora* R. Br. (Tetramelaceae)	A07376/LF+TW; A07377/SB; A07378/SW; A07379/RT	Bolikhamxay MPP
JMH 113	*Adenanthera pavonina* L. (Fabaceae-Mimosoideae)	A07380/LF+TW; A07381/SB; A07382/SW	Bolikhamxay MPP
JMH 114	*Fernandoa* cf. *adenophylla* (Wall. ex G. Don) Steenis (Bignoniaceae)	A07383/LF+TW; A07384/ST	Bolikhamxay MPP
JMH 115	*Uncaria sinensis* (Oliv.) Havil. (Rubiaceae)	A07385/LF+TW; A07386/ST	Bolikhamxay MPP

Plant part abbrevations: FL (flowers); FR (fruits); LF (leaves); PL (whole plant); PX (aerial parts); RT (roots); SB (stem bark); ST (stem); SW (stem wood); TW (twigs). [a]Traditional family names for each species listed are used in this paper; for current family names, please consult Tropicos (http://www.tropicos.org/), The Plant List (http://www.theplantlist.org/) or APG III (http://www.mobot.org/MOBOT/research/APweb/).

(MCF-7) (Soejarto et al., 2002; Kinghorn et al., 2003; Gyllenhaal et al., 2012; Zhang et al., 2016). Of these only HUVEC and hTERT-RPE1 cells are not human tumor cell lines, and only HL-60 is clearly not epithelial in origin. The NCDDG program utilized all of these cell lines as well as a number of additional ones (Kinghorn et al., 2003). For the cell lines utilized in the ICBG project, the cytotoxicity hit rate for Lao ethnomedical samples from plants ranged between 5 and 9%, with the exceptions of the non-epithelial leukemia cell line HL-60 (1.9%) and the breast cancer cell line MCF-7, as no bioassays were performed with this latter cell line for these samples (Gyllenhaal et al., 2012). No comparable meta- analysis in relation to the cell lines utilized was produced by the NCDDG, although a synthesis communicated significant insight into potential chemotaxonomic and plant anatomy-based relationships to activity hit rates and levels of cytotoxicity (Balunas et al., 2006).

Table 2. Plants from the Xiengkhouang medicinal biodiversity preserve with samples exhibiting notable cytotoxicity in HT-29 colon adenocarcinoma cells.

Species	Active sample (Corresponding voucher specimen)	Plant part(s) active	% HT-29 cell survival (2 µg/mL)	% HT-29 cell survival (20 µg/mL)
Cryptolepis dubia	A07194 (JMH 005)	Stem (ST)	100	48
Rubia argyi	A07196 (JMH 006)	Aerial parts (PX)	69	36
Reevesia pubescens	A07214 (JMH 021)	Fruits (FR)	84	24
Maclura tricuspidata	A07257 (JMH 044)	Leaves, twigs, and fruits (LF+TW+FR)	79	56
Millettia pachyloba	A07338 (JMH 094)	Stem (ST)	77	33
Gardenia annamensis	A07365 (JMH 107)	Leaves and twigs (LF+TW)	100	37

Table 3. Comparison of plants and plant parts active with their local employment in traditional medicine.

Active species	Plant part(s) active against HT-29 cells	Lao local name	Plant part(s) used in traditional medicine	Traditional use
Cryptolepis dubia	Stem (ST)	*Kheua en one*	Liana	Tonic for tendon and muscle
Rubia argyi	Aerial parts (PX)	*Kheua lin ma nai*	Whole liana	Fever, sore throat, kidney stone
Reevesia pubescens	Fruits (FR)	*Mai sa fay*	Root, stem	Gastritis
Maclura tricuspidata	Leaves, twigs, and fruits (LF+TW+FR)	*Kok nam thaeng*	Root	Kidney edema (swollen kidney), tonic for mother after giving birth
Millettia pachyloba	Stem (ST)	*Xa kheuy done*	Liana	Laxative
Gardenia annamensis	Leaves and twigs (LF+TW)	*Khai nao*	Stem	Stomachache

Anticancer bioprospecting from plants: Medical ethnobotany collection, biodiversity-based collection, and the continuum in between

Overall for the Lao ethnomedical plants on a per sample basis, the hit rate for cytotoxicity in cancer cells was higher whereas the “random” plants (from Vietnam) had a higher hit rate on a per collection basis in the ICBG (Gyllenhaal et al., 2012). This disjunction can potentially be explained in part through the fact that ethnomedical plant collection strategy often samples only the plant part used locally whereas the “random” plant collection strategy tends to sample as many plant parts per plant as can be managed, and therefore “random” collection provides more chances for each taxon to possess a sample bearing activity in one or more bioassays (Spjut, 2005; Gyllenhaal et al., 2012). For our expeditions outlined here overall, for those taxa with collected samples, a little over two (2.09) samples per taxon were obtained, some of which corresponded to plant parts used in local ethnomedicine and others of which did not. Interestingly, 50% (three) of the active samples were from plant parts employed from these taxa ethnomedically and the other 50% (three) were from plant parts not used ethno-medically to our knowledge. For each plant that was active, only one sample out of the 1 to 3 samples collected per taxon demonstrated significant cytotoxicity (Table 3). None of these plants bearing active samples was employed locally to treat cancer. In this context, these facts preliminarily suggest that the strategy of revisiting areas with documented ethnopharmacopeias, collecting as many samples as possible per plant harmonizes the benefits of “random” and ethnobotanical collection strategies for anticancer screening while mitigating their respective downsides.

Richard W. Spjut opines that while cytotoxicity to tumor cell lines and antitumor activity for a plant taxon cannot be known *a priori* based on one reported ethnomedical application versus another,

comparing literature review and ethnopharmacological field work in tandem with biological screening suggests in general that: (a) greater toxicity categories exhibit higher hit rates than all medicinal plants taken as one category and (b) certain categories had three (anthelminthics) to four (arrow poisons and homicidal agents) times the hit rates of plants screened from “random” collections (Spjut, 2005). Still it is not necessarily straightforward that maximizing cytotoxicity in prioritizing bioactive leads always catalyzes advancement in the US pharmaceutical pipeline, as evidenced by the development of anti-hepatitis C nucleoside analogues for instance, which was among the first preclinical pharmacological research to suggest that it may behoove scientists to emphasize leads that retain moderate activity while minimizing toxicity (Sluis-Cremer et al., 2009; Coats et al., 2014). Plants and other sources of bioactive metabolites may be ideally positioned to take advantage of the uncertain interplay between bioactivity and toxicity in the transition from preclinical to clinical evaluation of leads, given that they often produce a suite of closely related analogues. This is particularly salient for plants collected on the basis of use in local medicine, by which their safety for human administration is comparatively and more likely assured in contrast to other sources of bioactive compounds (combinatorial chemistry, microbes, etc.). While as a result of his meta-analysis Spjut furthermore advocates selectively mining medicinal plants for categorical, chemotaxonomic, and/or novelty-related reasons in ongoing screening efforts, he also admits that the faster pace and greater sample collection rate for “random” (biodiversity-based) collection expeditions is attractive (Spjut, 2005). This is interesting because his analysis includes a further admission of the hybridity of collection strategies (Spjut, 2005). This hybridity is bound up in multiple explanations as to why the “random” collection strategy should be punctuated in quotations since, for instance, phytogeography always and chemotaxonomy still often constrain the hit rate results of plants selected for sampling from a given area.

It should be noted that our Lao collection strategy for P01 anticancer screening resembles the pace and sample collection rate of the “random” strategy that Spjut outlines far more than the typical ethnobotany-driven or active sample re-collection expedition in his experience, with an average of ~40/day obtained between the two expeditions, and within the 1 to 3 active samples (out of 60 to 100 acquired) per day projected for a “random” collection effort (Spjut, 2005). This also implies that at the very maximum, a “random” collection effort might be expected to have twice the hit rate of these Lao P01 collection expeditions, while at the low end, such “random” screening could have only a third of the hit rate observed. As with “random” collection expeditions for anticancer screening, chemotaxonomic considerations had an impact on the samples that were collected (Spjut, 2005; Balunas et al., 2006). This principle along with related inherited wisdom from the National Cancer Institute and the NCDDG/P01 project experience informed our exclusion of certain taxa for sample collection throughout the expeditions. These built-in considerations help demonstrate that this ethnobotanically driven screening strategy implemented for these two Lao expeditions is not an example of ideological purity but rather of hybridity. It would be argued that this eclectic pragmatism contributes to the effectiveness of the innovative strategy as a viable screening paradigm, which is supported perfectly by the fact that twice the number of active hits was generated by broad collection of plant parts as would have been through collection solely of plant parts employed in local ethnomedicine.

Prior results and integrative programing in Laos and Vietnam as a predictor of future success and strategic considerations for medicinal plant bio-prospecting

The expansive and human-centric scope of the Vietnam-Laos ICBG has established and funded infrastructure bolstering traditional medicine through the creation of rural preserves and traditional medicine stations (TMS) in the Lao PDR (Riley, 2001; Sydara et al., 2014; Soejarto et al., 2015); permitted scholarly pursuit of botanical medicines described in Lao palm leaf manuscripts (Elkington et al., 2009); and thoroughly achieved the inventory and preliminary drug discovery lead investigation of the medicinal plants, and the floristic diversity as a whole, in these two countries (Soejarto et al., 2002, 2006, 2012; Sydara et al., 2014). This project’s legacy is an exemplar of the fact that medical ethnobotany, alongside such interdisciplinary subfields as historical ecology, landscape archaeology, and political economy (Balée and Erickson, 2006; Campbell, 2007; Scott, 2009), holds human agency intentionality in high esteem, being that they are crucial to value-added applications resulting from examination, discovery, and rediscovery of traditional knowledge. Indeed, human-environment interactions and the cultural transmission of natural and physiological observations are the bedrock underlying the endeavors of medical ethnobotany and ethnopharmacology, ensuring their value in perpetuity (Ott, 1998; Shepard, 2004).

Consideration of past plant collection practices and biological screening results in Vietnam and Laos to date should be able to guide the principles by which future expeditions are performed in the collection of plant samples for the P01 project in the Lao PDR. For instance, in the Vietnam-Laos ICBG, whereas cancer cell cytotoxicity hit rates for “random” samples from Vietnam and ethnomedical samples from the Lao PDR were relatively high, the ethnomedical samples from Vietnam had yielded a low hit rate (Gyllenhaal et al., 2012). It has

been suggested that the differences in these hit rates in ethnobotanical collections between the two countries is attributable to the following factors: (a) a greater proportion of accessions in Laos that were purported correctly by healers to treat cancer in particular and (b) a more specialized knowledge of medicinal properties of flora owing to the Lao healers' greater prominence and mastery (Gyllenhaal et al., 2012). While it has been demonstrated that this study strategy can lead to new bioactive leads for anticancer drug discovery, category-focused ethnobotanical collection for biological screening seemingly is a tractable and rewarding strategy for evaluating the medicinal potential of Lao plant biodiversity as well.

Although no bio-prospecting and isolation work from the Lao PDR has thus far proceeded to the stage of patent filing thereby meriting additional studies (unlike for Vietnam; Zhang et al., 2014, 2017), through the Vietnam-Laos ICBG, six medicinal plant taxa from around the country have been evaluated through the compound isolation stage and have been found to contain mostly novel phytochemicals bearing anti-tuberculosis (Elkington et al., 2014), anti-malarial (He et al., 2005, 2006; Libman et al., 2008; Ma et al., 2008); and cancer cell cytotoxicity activities (Zhang et al., 2004). With respect to anticancer bio-prospecting from the ICBG field work conducted from 1998 to 2012 in the Lao PDR, at least 34 species yielded 50 extracts with significant activity (IC_{50} < 20 µg/ml) in one or more cancer cell lines of a six cell line panel, which notably led to the isolated compounds asparacoside and 3''-methoxy-nyasol (IC_{50} > 4 µg/ml, < 10 µg/ml) from *Asparagus cochinchinensis* (Lour.) Merr. (Soejarto et al., 2012). Integrative research with plants sourced from the network of ten preserves now established in Laos, to search for unanticipated bioactivity, is only in the most incipient of stages. Given adequate resources, biological screening and new active compound discovery from higher plants in these diverse habitats of the Lao PDR could continue indefinitely for decades to come. The link between bio-prospecting results and the biodiversity of these preserves and the country overall is as yet somewhat confounded by the underexplored relationship of abiotic and biotic factors to the phytochemistry of higher plant taxa throughout the monsoon cycle and under local management. These dynamic preserves are liable to yield additional surprises for as long as interdisciplinary programs such as the P01 project are able to support additional expeditions and follow-up research on taxa of interest (Kinghorn et al., 2009, 2016).

Conclusions

The two initial P01 expeditions to the Lao PDR demonstrate that biological screening efforts, particularly anticancer bioprospecting, can proceed in areas with ancient and ongoing cultures of medicinal plant use, with the cooperation of local residents who protect, manage, and depend on the various forest habitats of Laos. Given the unique opportunity for direct collaboration with the Institute of Traditional Medicine (ITM) and some of the authors' involvement with healers and protected areas throughout Laos, it is fully expected that further expeditions will yield comparable or improved results. There are clear reasons why ethnobotanically motivated plant collection in areas rich in medicinal plant reliance can pay greater dividends for drug discovery efforts than other strategies. These rationales stem from the requirements of human safety, minimization of toxicity, and mitigation of related side effect profiles, as well as efficacious pharmacological activity (as vetted by longstanding use by local people), and from the pace of sample collection and turnover of expedition results in terms of the substantial timetables for biological screening and compound isolation work. The Bolikhamxay MPP and Xiengkhouang MBP, which were investigated during the winter dry season, are only two of the ten preserves established in the country. It is hoped that more of such preserves will be established in the future and maintained locally to bolster resilience in medicinal plant resource use and management, ensuring long-term protection of these forested regions throughout Laos.

Far more inventory work and sample collection expeditions to these preserves would be needed to truly exhaust the prospective bioactive leads from these ethnopharmacopeial treasure troves of traditional plant medicine, even for anticancer screening alone. Our interdisciplinary methodology and data analysis produce botanical and phytochemical leads not documented by local plant use patterns, but on the whole and verifiably anticipated by their value in ethnomedicine. The results of this research support the hypothesis that investigating plants known to be employed in local ethnopharmacopeias can produce promising starting points for natural product screening programs and pharmaceutical development, particularly as a first stage for anticancer drug discovery.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the guidance of Dr. Yali Fu, who executed an SDCR status application for collections-based research of the National Institutes of Health/National Cancer Institute P01 project (P01CA125066) in the Lao PDR. The status was awarded by the U.S. Department of State on September

9, 2015, which allowed for the collection of voucher specimens and samples in Laos with the collaboration of the Institute of Traditional Medicine, permitting material transfer to and biological evaluation in the USA. They also wish to acknowledge the ethnobotany, pharmacognosy, and chemistry staff of the Institute of Traditional Medicine for their involvement and support in the project. This research could not have proceeded without the broad participation of the ITM staff, and the authors are thankful for their tremendous effort and immense knowledge-base. They also wish to acknowledge the insights and efforts of their workers on these two expeditions, notably Mr. Buasy in the Xiengkhouang MBP and Mr. Bounyong in the Bolikhamxay MPP. Lastly, the participation of officials employed by the Food and Drug Divisions of the Provincial Health Departments of Bolikhamxay and Xiengkhouang was essential for these expeditions to proceed.

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academicJournals

Vol. 11(40), pp. 635-641, 25 October, 2017
DOI: 10.5897/JMPR2017.6460
Article Number: B944C7166462
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

In vitro antimicrobial activity and fatty acid composition throughgaschromatography-massspectrometry (GC-MS) of ethanol extracts of *Mauritia flexuosa* (Buriti) fruits

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Received 3 August, 2017; Accepted 5 October, 2017

In this study, the chemical composition of the peel and pulp of *Mauritia flexuosa* fruits were analyzed and the antimicrobial activity of ethanolic extracts from these fruits was evaluated using *in vitro* tests. Chemical composition analysis with gas chromatography-mass spectrometry (GC-MS) indicated the presence of saturated and unsaturated fatty acids. The peel extracts (ECBU) presented 54.41% and the pulp (EPBU) presented 94.05% of the saturated fatty acids lauric, myristic, palmitic, stearic, oleic and linoleic acids. The antimicrobial activities were performed using the diffusion and micro-dilution (MIC) methods. ECBU was active against the bacteria *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* at a concentration of 200 mg mL^{-1}, but it was not active against the yeasts *Candida albicans* and *Candida parapsilosis* using the diffusion method. The MIC results showed that ECBU was active against the tested bacteria at concentrations > 12.5 mg mL^{-1} and EPBU was active at concentrations > 25.0 mg mL^{-1}. This was probably due to higher sensibility of the method. The results indicated that the peel and pulp extracts of *M. flexuosa* present antibacterial activity and that ECBU is an especially promising potential candidate for the prospection of new pharmaceutical compounds.

Key words: *Mauritia flexuosa*, Buriti, anti-bacterial agents, fatty acids.

INTRODUCTION

The vast availability and indiscriminate use of antimicrobial compounds has led to a selection of micro-organisms that are resistant to these drugs. These drugs exert influence both in the patient under treatment and

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the ecosystem, with significant repercussions in the result of the disease and also in the increase in resistant environmental bacterial strains and species (Avorn and Solomon, 2000). In order to supply an increasing demand for new antimicrobial drugs, research on new sources of substances, including plants, has grown (Caetano et al., 2002). Bioactive compounds from plants have presented high specificity against a broad spectrum of bacteria (Dixon, 2001). The Cerrado and Amazonian biomes present 20% of all the biodiversity in the world (Calixto, 2005), which includes great diversity of plants with well-known therapeutic properties and chemicals that can be used in biological studies. *Mauritia flexuosa* L.f. (buriti) belongs to the Arecaceae family and is considered one of the most abundant oleaginous palms in Brazil, where it is native. The fruits of buriti are spherical or oval with seasonal fruiting (Storti, 1993), are rich in vitamin A and carotenoids which gives them their characteristic yellowish/reddish color (Albuquerque et al., 2003) and are traditionally consumed *in natura* (Barbosa et al., 2010). The commercialization of products from this palm tree in regions where it is native provides income for the local population and helps maintain the integrity of the "veredas" ecosystem, its main habitat. The indigenous Brazilian people call this species "the tree of life", due to the use of most of its parts, from the leaves to the root. Ribeiro et al. (2014) found 40 different uses for buriti among traditional native communities in Northwest Brazil. The studies of bioactive compounds with antimicrobial activities from buriti fruits are very rare. Buriti oil is reported as presenting antimicrobial properties as a soap formula (Soares et al., 2017). Koolen et al. (2013) and Batista et al. (2012) showed antimicrobial activity of extracts of leaves, trunk and fruits of *M. flexuosa*. Melhorança Filho and Pereira (2012) report antimicrobial activity against *Staphylococcus aureus* by seeds of two other Amazonian palms, *Eutherpe oleracea* and *Bactris gassipaes.* Barros et al. (2014) showed that buriti cream was effective in healing of skin lesions in mice. Due to the economic importance of *M. flexuosa* for indigenous Brazilian people, the objective of this study was to carry out *in vitro* antimicrobial activity tests of the ethanol extracts from the pulp and the fruit peel against human pathogens and to analyze the chemical composition of the fatty acids presented in gas chromatography coupled to a mass spectrometer. There are few studies on the antimicrobial activities of the chemical components (GC-MS) of the peel and pulp of this palm tree's fruits.

MATERIALS AND METHODS

Chemicals

Ethanol, Aluminum chloride ($AlCl_3$), Sodium chloride (NaCl), and Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mueller Hinton Broth and Sabour aud culture media were obtained from Kasvi (Curitiba, Paraná, Brazil). The water used in all analyses was ultrapure produced by a Milli-Q, Millipore system (Bedford, USA). Other reagents used in this study were of analytical grade.

Plant materials

Ripe fruits were collected from *M. flexuosa* (Figure 1a and b) in October 2015, in "vereda" ("veredas" are well-defined ecosystems that occur within the Brazilian Cerrado biome, and are characterized by the presence of buriti palm trees in semi-waterlogged conditions) site in the State of Tocantins, Brazil (9°58'2.078934"S 48°17'28.64502"W), at an altitude of 488 m. A voucher specimen of *M. flexuosa* (10.952) was deposited at the HTO herbarium of *Universidade Federal do Tocantins* (Federal University of Tocantins - UFT).

Sample preparation

The *M. flexuosa* fruit peels were removed manually after immersing the fruit in warm distilled water (40ºC), and were separated from the pulp using a stainless steel knife (Figure 1c to e). Thereafter, the materials were dried in an oven with air circulation (Fanem, São Paulo, Brazil) at 40°C for 48 h and crushed in a home processor (Arno, São Paulo, Brazil). Samples of approximately 10 to 30 g were weighed on a precision analytical scale (Shimadzu do Brazil, São Paulo, Brazil) and placed in cellulose cartridges in a Soxhlet apparatus with 200 mL of ethanol solvent (Vetec, 99.8% P.A.) for extraction over five h. In the end of the process, the solvent was removed using a rotary evaporator (Cienlab, São Paulo, Brazil) with a reduced pressure of 45°C. The crude extracts from buriti's pulp (EPBU) and peel (ECBU) were stored in a sterile bottle and refrigerated (10 to 15°C).

Gas chromatography–mass spectrometry (GC-MS)

In order to analyze the chemical compounds presented in the plant extracts, they were derivatized (esterification reaction) by acid catalysis of boron trifluoride in methanol with heating (Meher et al., 2006). Analyses were carried out using a Shimadzu GC/MS QP Model 2010 Ultra chromatograph equipped with an HP-5MS (30 m × 0.25 mm × 0.25 µm) fused silica capillary column. Standards for the GC–MS were saturated alkanes (C11 - C40) The program temperature for the standards used was 50°C (0 min); 5°C min^{-1} reaching 310°C (20 min), in which the retention time of $C_{11}H_{24}$ is 10.020 min and that of $C_{13}H_{28}$ is 15.535 min in Split mode: 1:25. The heating ramp had been programmed for a temperature range of 50°C (0 min); 5ºC min^{-1} up to 300°C (10 min) at a speed of 3°C min^{-1}. Injection temperature: 300°C; Interface temperature: 250ºC in Split mode: 1:25. Helium gas was used as a carrier gas at a speed of 1.2 mL min^{-1}. The energy of the electron was 70 eV and the temperature of the ion source was 250°C. The compounds were identified by comparing the mass spectrometer and their GC retention data with standards. Further identifications were made by comparing the mass spectrometer with those of the NIST-08 (National Institute of Standards and Technology) libraries and those cited in the literature (Adams, 2017).

Antimicrobial assays

ATCC-type strains (American Type Collection Culture) were kindly provided by collection from the National Institute for Quality Control in Health at the Oswaldo Cruz Foundation (INCQS/FIOCRUZ – Rio de Janeiro, Brazil). The used bacteria used were: *Enterococcus faecalis* (ATCC 4083), *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 27853) and the yeasts used were: *Candida albicans* (ATCC 10231) and

Figure 1. *Mauritia flexuosa* is a palm tree that grows in and near swamps and other wet areas; (a) ripe fruit (b) fruit immersed in water (c) peeled fruit (d) and (e) shells separated for drying.
Source: Photos by the author.

Candida parapsilosis (ATCC 22019), microorganisms that are usually recommended for use in antimicrobial assays (Alves et al., 2008; Silva et al., 2012).

Antimicrobial sensitivity testing

The antimicrobial assays were performed in triplicate using the well diffusion method (CLSI, 2012) in Petri (140 × 15 mm) dishes with 50 mL of Muller Hinton Agar medium for bacteria and the same amount of Saboraud Agar medium for the yeast tests. Inoculum solutions were prepared using 3 to 4 colonies of the isolated strain in plates and diluted in 0.85% saline solution before reaching the corresponding turbidity of 0.5 on the McFarland scale (CLSI, 2003); that is, around 1.5×10^8 Colony Forming Units ($CFU.mL^{-1}$) of bacteria and 2.0×10^6 CFU mL^{-1} (Pelissari et al., 2010) of yeasts. A 10% solution of Dimethyl sulfoxide (DMSO) was used as the negative control, and 30 µg mL^{-1} of Fluconazole for the yeasts or 30 µg mL^{-1} of Chloramphenicol for the bacteria was used as the positive control. The solutions containing the inocula were swabbed on the surface of the media and the wells were made with a sterile cork borer. The wells were then filled with 50 µL of the tested extract diluted in 10% DMSO at concentrations of 200, 100 and 50 mg mL^{-1}, and with the positive and negative controls. After 24 h of incubation at 37°C (bacteria) and 25°C (yeasts), the microbial growth inhibition halos were measured in millimeters with a digital caliper.

Determination of the minimum inhibitory concentration (MIC): Determination of the minimum inhibitory concentration (MIC) was done using the broth microdilution technique as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Lima et al., 2006). The tests were performed in a “sensitive microtiter” plate with 96 sterile wells only for microorganisms that presented inhibition in the well test (*E. faecalis*, *E. coli*, *S. aureus* and *P. aeruginosa)*. Initially, 100 µL of Muller Hinton growth medium was added to each well, followed by the extracts that were added by performing serial dilution as recommended by Benfatti et al. (2010), thus obtaining a range of concentrations of the pulp or peel extracts (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 $mg.mL^{-1}$). A solution of 2000 µg mL^{-1} of Chloramphenicol was used as the positive control, leading to serially diluted concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8 µg mL^{-1}. The negative control was 10% DMSO. Bacteria viability was tested using serial dilutions from a starting solution of 10^7 CFU mL^{-1}. In addition, control of media sterility was also executed. The 5 µL inoculum of the 10^7 CFU mL^{-1} bacterial solution was added to all except the sterility control wells. The plates were covered with plastic film and incubated at 37°C for 24 h. After the incubation period, 30 µL of a 1% aqueous reazurine (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) solution was added to each well for 1 h. A resulting blue color in the well was read as growth inhibition and a reddish pink as non-inhibition.

RESULTS

Extract yields

The yield of the pulp extract (EPBU) was 14.13% and the

Table 1. Fatty acid composition (%) of the ethanol extract from *Mauritia flexuosa* peel (EPBU) and pulp (ECBU).

Fatty acid composition	ECBU % area	EPBU % area
12:0 lauric acid	38.52	84.08
14:0 myristic acid	-	3.97
16:0 palmitic acid	15.20	2.02
18:0 stearic acid	1.69	3.98
18:1 oleic acid	41.17	5.56
18:1 trans-11 vaccenic acid	0.77	-
18:2 linoleic acid	2.65	0.39

Table 2. Mean diameter of growth inhibition (in millimeters (mm)) of bacterial strains in susceptibility tests using the ethanolic extracts ECBU and EPBU (concentration: 50, 100 and 200 mg mL^{-1}) from *M. flexuosa* fruits.

Microorganism	Diameter of the inhibition halo (mm)					
	ECBU (mg mL^{-1})			EPBU (mg mL^{-1})		
	50	100	200	50	100	200
E. faecalis	9.38 mm± 0.267	11.23 mm ±0.416	12.88 mm ±0.181	-	-	-
E. coli	-	11.63±0.559	14.22 ±0.498	-	-	-
S. aureus	10.55 mm ±0.280	12.61 mm ±0.200	15.50 mm ±0.434	-	-	-
P. aeruginosa	-	-	9.56 mm ± 0.223	-	-	-
C. albicans	-	-	-	-	-	-
C. parapsilosis	-	-	-	-	-	-

ECBU = Ethanolic extract from *M. flexuosa* fruit peel, EPBU = Ethanolic extract from *M. fleuxuosa* fruit pulp.

yield of the peel (ECBU) was 22.30%.

Fatty acid determination by gas chromatography

The values obtained by gas chromatography for the chemical composition of fatty acids in the crude extracts are presented in Table 1. The ethanolic extracts of *M. flexuosa* fruit peels contained both saturated (55.41%) and unsaturated fatty acids (44.59%). The saturated fatty acid was primarily lauric (38.52%) acid, while unsaturated fatty acids included oleic (41.17%) and linoleic (2.65%) acids. The ethanolic extract of the pulp had a high content of saturated fatty acids (94.05%) and unsaturated fatty acids (5.95%). Saturated fatty acids in pulps included lauric (84.08%), myristic (3.97%) and stearic (3.98%) acids, and unsaturated fatty acids including oleic (5.56%) and linoleic (0.39%) acids.

Antimicrobial activity of crude extracts

The antimicrobial activity test was performed with the crude ethanolic extracts ECBU and EPBU from *M. flexuosa* (Table 2) in which EPBU showed no inhibition halo against the bacteria tested. The extract ECBU presented an inhibition halo ranging from 0 to 15.5 mm for all bacteria at a concentration of 200 mg/mL. The largest inhibition halo occurred against *S. aureus* and the smallest against *P. aeruginosa*. At a concentration of 100 mg/mL, all bacteria were inhibited except *P. aeruginosa*. The extract was able to inhibit *E. faecalis* and *S. aureus* at concentrations as low as 50 mg/mL, but was not able to inhibit the other tested strains.

Minimum inhibitory concentration (MIC)

The MIC results from the extracts ECBU and EPBU are shown in Table 3. The used extract concentrations used in the test ranged from 50 to 0.39 mg/mL. The ECBU extract presented an MIC of 12.5 mg/mL against *E. faecalis*, 25 mg/mL against *S. aureus,* and 50 mg/mL against other tested bacteria, with an inhibitory response in lower concentrations than EPBU, which had an MIC between 25 mg/mL against *E. coli,* and 50 mg/mL against the other tested bacteria.

DISCUSSION

The ethanolic extracts obtained from the peels and pulp

Table 3. Minimum inhibitory concentration (MIC) in mg/mL of crude ethanolic extracts from the peel (ECBU) and the pulp (EPBU) of *M. flexuosa* with antimicrobial activities.

Crude extract	*E. faecalis*	*E. coli*	*S. aureus*	*P. aeruginosa*
ECBU	12.5	50	25	50
EPBU	50	25	50	50

of *M. flexuosa* fruits were shown to be available and easily obtainable source of antimicrobials active against a range of bacterial strains. The Soxhlet system was chosen to obtain the extracts because it is a standard method in which the temperature and nature of the solvent determine and favor the extraction efficiency of the active compounds. Ethanol was the solvent chosen because it is affordable, comes from a renewable source, has low toxicity and is capable of extracting a wide range of polar compounds and some non-polar compounds (Bastos et al., 2010). EPBU yield was 14.13%, which is lower than values of 23.55% found in the literature (Carvalho et al., 2011) probably because the extraction method used hexane as the solvent instead of ethanol for 12 h in a Soxhlet extractor. On the other hand, the ECBU yield of 22.30% was greater than that found by Fuentes et al. (2013) of 13% using hexane as the solvent over 8 h.

The differences in yields obtained may be related not only to the nature of the solvents, but also to other factors such as temperature, soil type, humidity, and general sanity of the tree, etc. which can cause the plant to produce different substances. For example, Vasquez-Leon et al. (2017) showed that bioactive compounds in *Moringa oleifera* Lam. leaves are influenced by climatic factors, soil, and tree age. Milanez et al. (2018) discussed that buriti fruits harvested at different stages of ripening produced different quantities of total phenolic compounds, especially among fruits harvested at the ripened stage, where the levels of these compounds were higher.

The comparison between extracts obtained using ethanol and hexane shows that the percent of saturated fatty acids (55.41%) in ethanolic extracts of ECBU was lower than that extracted from the same fruit biomass when using hexane as the solvent (59%) (Forero-Doria et al., 2016). However, the percent of unsaturated fatty acids of ECBU (44.59%) was higher than what is reported by Darnet et al. (37.9%) (Forero-Doria et al., 2016), using hexane as the solvent. The percent of lauric acid in the ethanolic extract was higher (38.52%) than that obtained using hexane as a solvent (0.7%) (Fuentes et al., 2013). The obtained values for oleic acid (41.17%) and linoleic acid (2.65%) from ECBU were similar to the ones shown by Fuentes (2013), which has 33.4% for oleic acid and 3.7% for linoleic acid. Extraction using ethanol is a viable means of obtaining compounds from *M. flexuosa* fruits, especially the unsaturated fatty acids.

EPBU presented a higher percent of saturated acids (94.05%) than the values found in the literature [21.9% (Darnet et al., 2011) and 21.76% (Manhães and Sabaa-Srur, 2011)] and a lower percent of unsaturated acids (5.95%) compared to the values obtained for the hexane-extracted substrate (78.01 and 78.18%) (Manhães and Sabaa-Srur, 2011). The percent of oleic acid (5.56%) in ethanol-extracted EPBU was below what is commonly found in buriti pulp and lower than in hexane-extracted oil [75.7 and 73.32% (Manhães and Sabaa-Srur, 2011)]. The higher concentration of saturated fatty acids in the two ethanolic extracts (ECBU and EPBU) compared to extracts obtained using hexane is probably explained by the temperature increase during ethanol extraction (P.E. 78.37°C) as compared to hexane (68°C), which favored the extraction of the saturated compounds that are more resistant to oxidation and more stable at higher temperatures.

Antimicrobial activity tests were carried out with the agar dilution method that is widely used, since it presents simple execution and low cost, and could easily demonstrate the spectra of activity for both of the tested extracts. ECBU demonstrated activity against both G+ (*E. faecalis* and *S. aureus*) and G- strains (*E. coli* and *P. aeruginosa*), which indicates broad spectrum inhibitory activity against bacteria. However, it did not show activity against the yeasts tested (*C. albicans* and *C. parapsilosis*). The literature (Batista et al., 2012) reported an inhibition activity for the *M. flexuosa* pulp extract obtained with hexane extraction against *S. aureus* ATCC 6538. Silveira et al. (2005) showed that both ethanolic and hexanic extracts of *M. flexuosa* fruits were active against *S. aureus* and *P. aeruginosa*, but did not significantly inhibit *E. coli*.

Huang et al. (2011) demonstrated that fatty acids exhibit patterns of inhibition against oral bacteria with specificity that relates more to the bacterial species than the general structural characteristics of the microorganisms. This study also showed that fatty acids were much less effective against *C. albicans* than the oral bacteria, with effectiveness limited to hexanoic, octanoic, and lauric acids (Huang et al., 2011). We were not able to correlate the fatty acid composition to the halo of antimicrobial activity of the fruit since crude extracts were used for the testing of antimicrobial activity. Further studies of the antimicrobial activity of the combined or isolated fatty acids detected are needed to allow correlation of inhibition zone and fatty acid composition. It is also possible that the inhibition may be correlated not to a specific compound but to conjugated groups. Sugar

based surfactants conjugated with fatty acid chains are an emerging broad group of highly biocompatible and biodegradable compounds with established and potential future applications in the pharmaceutical, cosmetic and food industries. Lucarini et al. (2016) showed that synthetic lactose palmitoleate and lactose nervonate were shown to exhibit antimicrobial activity versus eight pathogenic species belonging to G+ and G- microorganisms and fungi.

EPBU showed no activity against the bacteria when tested with the well diffusion method. This result is different from (Mekonnen et al., 2016) probably because conditions in this experiment such as the extraction solvent and the microbial species and strains differed from other studies. The same EPBU extract presented a positive result in the MIC test and this may be related to the fact that this method allows for greater solubility of polar compounds (Miranda-Arámbula et al., 2017) that are present in the extract and better dispersion favoring interaction with the tested microorganisms (Valgas et al., 2007). It is also approximately 30 times more sensitive than the other methods described in the literature (Ostrosky et al., 2008). The MIC is widely used for simplicity, low cost, reproducibility, sensitivity and for using a minimum amount of reagents, which allows for a greater number of replicates, increasing the reliability of the results and leaving a permanent record.

The presence of fatty acids in *M. flexuosa* extracts could have been contributed to their antimicrobial activity. The antimicrobial effect of these acids occurs because they affect the cell wall, interfering with mechanisms of bacterial virulence such as the prevention of biofilm formation and inhibition of toxin and enzyme production (Ogidi et al., 2015). The entire process of investigation that included information retrieval, botanical identification of the species, research and experimentation provides subsidies for the production of efficient and inexpensive products. In addition, it could also be a social and economic reinforcement for families in the regions where the fruit is found and widely consumed.

Conclusion

Buriti (*M. flexuosa*) fruits and their products present great economic and social importance in the geographic areas where this plant is autochthonous. The obtained ethanolic extracts from the pulp and peel of these fruits showed antibacterial activity against the human pathogens studied. The gas chromatographic analysis (GC-MS) identified the fatty acids: lauric, myristic, palmitic, stearic, oleic and linoleic. Therefore, this study concludes that ECBU and EPBU present potential for pharmaceutical and technological applications due to the presence of bioactive compounds with antibacterial activity and has brought forward new information on the biotechnological potential of this Brazilian palm tree.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ECBU, Ethanolic extract of Buriti bark; **EPBU,** ethanolic extract of Buriti pulp; **MIC,** minimum inhibitory concentration; **G+,** gram positive; **G-,** gram negative; **GC-MS,** gas chromatography coupled to mass spectrometer; **DMSO,** Dimethylsulfoxide; **ATCC,** American type collection culture; **CFU,** colony forming unit; **CLSI,** Clinical and Laboratory Standards Institute.

ACKNOWLEDGEMENTS

The authors are grateful to the Chemistry Department, Center of Technological Sciences (CCT) from Santa Catarina State University (UNIDESC) for the use of its premises for GC/MS analyses and to Edmar Martendal Dias de Souza for the support. This study was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) (AUXPE-PRO-AMAZONIA-3312/2013/process no. 23038.010315/2013-66).

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