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Investigator's Report for Scientific Research: Plant Collections from Puerto Rico for Antitumor Screening DRNA: 03-1C-014

Principal Investigator: Richard Spjut

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OBJECTIVES

To obtain 350 samples for the National Cancer Institute's antitumor screening program, represented by root, bark, twig, and leaf from a broad diversity of taxa with emphasis on species confined to the Caribbean Region.

SUMMARY OF ACCOMPLISHMENTS

Three-hundred and seventeen (317) plant samples were collected in Puerto Rico during February 2004 from 66 species represented by 54 genera and 38 families; 43 species were from the Maricao State Forest Reserve, 19 from the USFS Caribbean National Forest, and 4 from private land. Species were sampled based on phytogeographical criteria, generally Caribbean in distribution as represented by 60 species of which 40 are endemic to the Islands (West Indies). These data are shown in a table (Appendix I) along with data on the number of extracts tested in each genus as reported by the NCI in their RFP (2000), compared to an estimate on the number of species in the genus from J. Mabberley's *The Plant Book* (2nd ed., 1997). Samples from three genera are new to the NCI screen.

BACKGROUND

Under a reimbursable agreement, the World Botanical Associates (WBA) supplies the National Cancer Institute (NCI) with 1,000 samples of plants each year from the United States and Territories in search of new anticancer drugs. Samples of selected plant parts, or the entire plant, are collected in quantities to yield 300–500 g dry weight for extraction and testing against 60 different cell lines of cancer. Examples of anticancer drugs discovered from the NCI screening of plant extracts are taxol from stem-bark of *Taxus brevifolia*–originally collected in the state of Washington, and derivatives of

camptothecin from stem or twig, leaf and fruit of *Camptotheca acuminata*—a plant native to S China that was collected at a USDA plant introduction station in Chico, California (Perdue et al. 1970).[1]

The WBA follows a systematic approach based on phytogeographical relationships with limitations in regard to a RFP (Request for Proposal) for which we submitted replies to the NCI in Nov. 2000 and Apr. 2001. The limitations required consideration to 5,000 samples already collected within the United States—by the Morton Arboretum (William Hess, PI, 1997–2001), and 96 extracts from samples obtained by the New York Botanical Garden from Puerto Rico. Our analysis—in response to the NCI RFP—revealed most samples were from deciduous forests of the eastern United States. In our response to the RFP (2000), we proposed to collect 700 samples from Puerto Rico in YR V (2006). This schedule was moved ahead at the request of NCI. For budgetary reasons, we also decided to spread the collecting over a period of two years—in two 30-day visits, 350 samples for each trip.

Spjut (1985),[2] in a review of the NCI plant collection program, recognized 58 floristic regions for the world. Puerto Rico lies within the West Indies Floristic Region. Our selection of plants in Puerto Rico also included species that occurred in the Central American Floristic Region (Spjut 1985) and South American countries bordering the Caribbean Sea. We refer to this broader region as the Caribbean Region. This was the geographic limitations we generally adopted in our selection of samples in Puerto Rico for the NCI antitumor screening. We felt that collecting plants with a geographical distribution within the Caribbean Region would minimize duplication of collections for the NCI based on those previously collected by the Missouri Botanical Garden in Africa and Madagascar, the New York Botanical Garden (NYBG) in tropical America, and the University of Illinois at Chicago in tropical SE Asia.

The Principal Investigator (PI, Richard Spjut) identified plants in the field using manuals on the local flora before sampling. Vouchers were prepared for all species sampled. Identifications of vouchers are reviewed by Roger Sanders except Asteraceae by Guy Nesom, Botanical Research Institute of Texas. Duplicates are deposited in the US National Herbarium (US), WBA (World Botanical Associates), and in Puerto Rico.

History of the NCI Natural Products Screen

The NCI has been screening plant extracts for novel antitumor agents since 1956,[3] although NCI had conducted experimental antitumor screening projects on selected plants as early as 1952.[4] A primary impetus for this program is the discovery of the vinca alkaloids in the Madagascar periwinkle (C*atharanthus roseus*), and the lignan,

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[1] R. E. Perdue Jr. , R. L. Smith, M. E. Wall, J. Hartwell & B. Abbott, *Camptotheca acuminata Decaisne [Nyssaceae] Source of Camptothecin, an Antileukemia Alkaloid*, USDA Tech. Bull. 1415, 1970.
[2] R. W. Spjut, *Limitations of a Random Screen: Search for New Anticancer Drugs in Higher Plants,* Economic Botany 39: 266-288, 1985.
[3] Abbott, *Bioassay of Plant Extracts for Anticancer Activity*, Cancer Treat. Rep. 60: 1007–1010, 1976.
[4] M. Belkin & D. B. Fitzgerald, 1952. *Tumor-damaging capacity of plant materials I. Plants used as cathartics*. J. Nat. Cancer Inst. 13: 139–155. II. (With M. D. Felix), 1952. *Plants used as diuretics*. J. Nat. Cancer Inst. 13: 741–744. 1953—III. *Plants used as pesticides*. J. Nat. Cancer Inst. 13: 889–893. IV. *Conifers*. J. Nat. Cancer Inst. 13: 895–903, and V. *Miscellaneous Plants*. J. Nat. Cancer Inst. 14: 607–625.

podophyllotoxin, in may-apple (*Podophyllum peltatum*). Compounds from both have led to drugs currently used for treating cancers, vincristine and vinblastine (vinca alkaloids) for leukemia, Hodgkin's lymphoma, breast and colorectal cancers, and derivatives of podophyllotoxin (e.g., etoposide) for bronchial, lymphoma and testicular cancers.

The antitumor compound, camptothecin, mentioned earlier, was discovered from pursuing a preliminary active among ~1,500 plant extracts randomly screened by the NCI during 1956–1960 (Shephartz 1976).[5] About 1,000 of these extracts were obtained by J. Hartwell (NCI) from the USDA Plant Steroidal Investigations. Among these was reportedly an extract from a leaf sample of *Camptotheca acuminata,* a plant native to southern China that was cultivated at Chico, California (Perdue et al. 1970, loc. cit.), one of four plant introduction stations where the USDA had collected and extracted plant samples. The isolation and characterization of camptothecin was carried out by Monroe Wall's group at Research Triangle Institute (Wani & Wall, J. Org. Chem. 34: 1364, 1969) from large recollections of "stem" (ws-sb) since further collections of leaf samples failed to reconfirm in LE (Perdue et al., loc. cit.), while an extract from a fruit sample had also showed confirmed activity in LE (July 1962). Recollections were obtained from cultivated plants at Chico and other areas in southern California. Derivatives of camptothecin (topetecan and irinotecan)—that have since been discovered—have led to FDA approved drugs for use in treating lung, ovarian and colorectal cancers.

From 1960 until 1982, the NCI screened ~120,000 extracts from ~35,000 plant species that were supplied by the ARS (~58,000 samples) and other institutions (Spjut 1985, loc cit.). Although the NCI acquisition of plant samples was regarded random, plant procurement always had taxonomic guidelines (Spjut 1985, loc. cit.).

Many active plants discovered by the NCI show apparent correlation with a broad spectrum of therapeutic uses reported in folk medicine (Spjut & Perdue1976);[6] however, the cost to selectively collect such samples is at least 10 times that of a phytogeographical approach (Spjut 1985, loc. cit.). Spjut and Perdue (1976, loc. cit.) had concluded that sampling the available flora was the most cost effective approach to discovery of novel biologically active compounds, which would include plants generally not reported to have medicinal uses (e.g. *Camptotheca*).

The extraction procedures and bioassays were also modified to be more selective in discovery of new anticancer compounds. Major changes included introduction of new tumor assays, extracting out tannins, and dropping bioassays that were sensitive to ubiquitous compounds.[7] Between 1969 and 1980, two prescreen assays were primarily

[5] Shephartz, *History of the National Cancer Institute and the Plant Screening Program*; Cancer Treat. Rep. 60: 975–977, 1976.

[6] R. W. Spjut & R. E. Perdue Jr., *Plant Folklore, A Tool for Predicting Sources of Antitumor Activity?* Cancer Treat. Rep. 60: 979–985.

[7] Tumors initially used in the screen were the L-1210 and two others randomly selected from a battery of 23 systems in which sarcoma (SA) 180 and adenocarcinoma (CA) 755 were more frequently used (Abbott 1976, loc. cit.). Activity in the L-1210 (also LE) was very rare, about one in 12,000 according to D. Statz & F. Coon, *Preparation of Plant Extracts for Antitumor Screening*, Cancer Treat. Rep. 60: 999–1005, 1976 however, extracts from some plants (e.g., *Taxus brevifolia*) that were initially inactive against LE showed LE activity upon fractionation. Almost all plants that showed LE activity advanced to clinical studies (see also Perdue, J. Nat. Prod. 1982).

employed, the *in vitro* 9KB and *in vivo* P-388.[8] More than 1,000 novel active compounds were probably discovered; Hartwell (1976) summarized chemical and activity data on 322 compounds.[9]

A major discovery during this period was taxol from stem-bark of *Taxus brevifolia.* Taxol (paclitaxel) is currently used to treat ovarian, breast and other cancers. The stem-bark sample that initially showed activity was collected in 1962 by an ARS botanist, Arthur S. Barclay, who was assigned (by Robert Perdue, Quintin Jones and Carl Erlanson, New Crops Research Branch) to collect in the western US. It was one of ~ 500 species sampled by Dr. Barclay, with the assistance of several students, in the Pacific Northwest (California, Oregon, northwestern Nevada, and Washington) during a three month period, June to August[10] in which species were collected as encountered in quantity to yield at least 1 pound (or 500 g) dried, except those already collected.

In 1986, the NCI redeveloped its natural products screening. Instead of a collaborative agreement with the ARS, the NCI awarded contracts to botanical institutions with expertise in the geographic areas where collections were to be obtained, the Missouri Botanical for Africa, the New York Botanical Garden for Tropical America, and the University of Illinois at Chicago for SE Asia. The NCI has obtained approximately 60,000 samples since 1986.

The new NCI antitumor screen with "60 different human tumor cell lines" became routine in 1990 (Boyd and Paull, Drug Development Research 34: 91–109. 1995). However, the major novel discoveries so far are antiviral (Cragg et al. 1996).[11]

Plants Screened for Antitumor Activity from Puerto Rico

Spjut (1985, loc. cit.) indicated that NCI had screened a limited number of plant samples from the West Indies Floristic Region. Species reported with confirmed antitumor activity from Puerto Rico are listed in Appendix II. With exception to one sample, they were originally procured in 1967 by the ARS botanist, Lloyd Spetzman, and USDA personnel stationed at the plant introduction station in Mayaguez. Another ARS

[8] In 1966, the NCI screen consisted of Walker intramuscular (WM) 256, LE and KB (Eagle) cell culture (KB). In 1969, P-388 leukemia (PS) was substituted for the LE and WM (WA) was dropped (Hartwell 1976, loc. cit.). The latter was sensitive to tannins, which by 1969 were being extracted out, and also phytosterols; both classes of compounds accounted for ~ 40% of all actives in WM (Perdue, ARS Cancer Program Guide, Oct-09-1970). Sometime around 1980, the Astrocytoma (ASK) was introduced to the NCI prescreen.

[9] J. Hartwell, *Types of Anticancer Agents Isolated from Plants*, Cancer Treat. Rep. 60: 1031–1067.

[10] Although sb of *Taxus brevifolia* was collected in August 1962, it was first sent back to Beltsville MD for identification and accessioning (assigned a lab PR number and weighed), before it was shipped to the extraction facility ("WARF") on Jan. 9, 1963 (date stamped on USDA accession record), and it was not until July 1964 when it was reported as a confirmed active (in KB).

[11] Significant antiHIV agents discovered include (+)-Calanolide A and (-)-Calanolide B (costatolide) from *Calophyllum lanigerum* and *Calophyllum teysmanii*, respectively, in Sarawak, Malaysia; conocurovone from *Conospermum incurvum* in Western Australia; michellamine B from *Ancistrocladus korupensis* in Cameroon; and prostratin from wood of *Homolanthus nutans* in Western Samoa. Cragg, G.M., J.E. Simon, J.G. Jato, and K.M Snader. 1996. *Drug discovery and development at the National Cancer Institute: Potential for new pharmaceutical crops.* p. 554-560. In: J. Janick (ed.), Progress in new crops, ASHS Press, Arlington, VA.

botanist, Sandra Saufferer, obtained general samples and recollections from Puerto Rico in 1980, adding one new active, *Daphnopsis philippiana* tw-lf. It might be noted that during the 1970's, the Plant Introduction Station in Mayaguez was a primary site for the cultivation of potentially new tropical crops such as *Brucea antidysenterica* (from Kenya), *Maytenus buchananii* (from Kenya), *Tripterygium wilfordii* (from Tawian) and other species that were sources of new anticancer compounds.

Most active species from Puerto Rico (Appendix II) are widespread in geographic distribution, 24 out of 31 species (Spjut 1974, ARS memo dated Feb. 6, and pers. comm. with Spetzman, copy provided in RFP Reply, Apr 2001). These are not truly representative of the Puerto Rico flora. However, it should be recalled that camptothecin was discovered from a random screening of plant samples collected at the USDA plant introduction stations in mainland USA, and it would seem that an attempt was being made to repeat this type of success in Puerto Rico.

Collecting samples from botanical gardens and introduction stations, however, poses limitations as to what plant parts can be collected. Root and bark samples, the parts mostly likely to show activity, aside from flower and fruit that are rarely available, are generally not collectable from plants cultivated under such circumstances. Thus, it is not surprising to find these kinds of samples lacking from Puerto Rico (Appendix II). The samples collected from the former USDA plant introduction station at Mayaguez were mostly from branches, divided into woody stems (ws-sb) and leafy (tw-lf) parts, the latter indicated as "st-lf" in Appendix II.

During the early to mid 1970's, the ARS Medicinal Plant Resources Lab increasingly procured samples for the NCI by subcontracting rather than by staff, except in East Africa where the leader, Robert Perdue, Jr., had been conducting field work (Perdue 1976). [12] Dr. Perdue continued to send lab botanists to East Africa because of the relatively low cost in labor; in those days, one could hire an African laborer for $1.00 per day. Because many active species from Puerto Rico could be obtained elsewhere, Dr. Perdue felt it was not really necessary to follow-up on recollections from Puerto Rico.

However, Dr. Perdue was replaced by Jim Duke in May 1978 as leader of the Medicinal Plant Resources Lab. with A. S. Barclay as Principal Investigator of the NCI procurement of plant samples; the lab. name was later changed—at the request by Dr. Duke—to Economic Botany Laboratory. An ARS botanist, Sandra Saufferer, subsequently conducted field work in Puerto Rico in 1980 for recollections of samples originally obtained by Lloyd Spetzman. The reports on the isolation of active agents are not readily known to the PI. Other recent reports—unrelated to the NCI program—include one on *Acalyphya wilkesiana* (Euphorbiaceae) from which an aqueous extract induced "apoptosis" and generated "active oxygen intermediates" in "granulocytes". [13]

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[12] R. E. Perdue, Jr. 1976. *Procurement of Plant Materials for Antitumor Screening*. Cancer Treat. Rep. 60(8): 987–998.

[13] A.K. Taraphdar, M. Roy, and R. K. Bhattcharya. 2001. *Natural products as inducers of apoptosis. Implication for cancer chemotherapy and prevention.* Dept. Environ. Carcinogenesis & Toxicol.

Left–*Gonzalagunia hirsuta* (Rubiaceae) and right–'Queens Flower', *Lagerstroemia speciosa* (Lythraceae), Caribbean National Forest. Samples were collected of *Gonzalagunia* but not of *Lagerstroemia* because the PI suspected the latter had been thoroughly screened from samples collected elsewhere. *Lagerostroemia* includes the crepe myrtle (*L. indica*) from which extracts of st-lf from Georgia and of sd (seed) from Italy were active in SA. Extracts from samples of other *Lagerostroemia* species, *L. calyculata* sb from Thailand and *L. macrocarpa* sb from Thailand were active in KB. An extract from *L. speciosa* lf (source unknown) was active in PS. Activity in SA is probably due to sitosterol. Other compounds in *Lagerostroemia* include the triterpene colosolic acid that is being investigated for treating type II diabetes. Species of *Gonzalagunia* are reported to have alkaloids, and one species has shown activity against snake venom.

Here it might be noted that extracts from wood sap samples of *A. diversifolia* and *Acalypha* sp. were active in PS, and that "oil" from *A. lanceolata* was active in WA.

Thus, it would appear that relatively few indigenous plants to Puerto Rico were screened by the NCI (Spjut 1985), and what was screened from there was mostly introduced species in which the parts collected were not those most likely to show activity.

Furthermore, the RFP (NCI 2000) indicated only 96 extracts had been screened from Puerto Rico (since 1986). The question as to whether we should obtain a printout of the species screened to avoid duplication seemed somewhat inconsequential to the PI. He realized that the NCI had already been screening samples from Mexico, Belize, Costa Rica, Bolivia, Ecuador, and other areas in tropical America where samples were largely supplied by the New York Botanical Garden (NYBG). To thoroughly avoid duplication would require an in-depth review of all plants screened from tropical countries. This was not in our budget. Assuming that the data in the NCI RFP is based on two extracts for each sample, only 48 samples may have been obtained from perhaps as few as 8 species (6 samples per species) to as many as 48 species (one sample per species). And since botanists from other institutions generally collect what is readily available for the NCI, it seemed that duplication would be less likely in our phytogeographic approach. An indication as to the extent of previous collections for genera we collected in Puerto Rico can be seen by the number of extracts tested in each genus shown in Appendix I.

Chittaranjan National Cancer Institute, Kolkata, India. Review Article
http://www.ias.ac.in/currsci/jun102001/1387.pdf

Collecting Samples in the Maricao State Forest Reserve. Richard Marin on left and Chief Forester, Adrian Muniz, on right in both photos, in left photo separating twigs from leaves and right photo showing use of a chain-saw to cut-up a ws (wood of stem with bark removed) sample.

The WBA Plant Collections from Puerto Rico, February 2004

Richard Marin obtained the plant collecting permits from Puerto Rico. Cooperation from all authorities in Puerto Rico was excellent. We collected mostly in the Caribbean National Forest (El Yunque) and Maricao State Forest Reserve.

While awaiting final approval from the USDA Forest Service for collecting in the Luquillo (Caribbean) National Forest, we identified 30 species in a coastal forest near Playa del Yunque north of Rio Grande (along the NE coast of PR). Upon review of the geographical distribution of species in our references that included Little, Woodbury and Wadsworth two volumes on the *Trees of Puerto Rico and the Virgin Islands*, and five volumes of Alain Liogier's *Descriptive Flora of Puerto Rica and Adjacent Islands*, we considered only two of the species acceptable for sampling, a fern native to Florida, *Acrostichum daenifolium*, and a tree, *Tabebuia heterophylla* (Bignoniaceae) found in Florida, Puerto Rico and the Virgin Islands. The Morton Arboretum, who collected in Florida under the previous contract for the US mainland, did not obtain samples of these species. It should be noted that if we had we followed the usual "bio-prospecting" approach, we could have easily obtained more than 100 samples from this beach forest. We rejected 93% of the species we identified in this coastal forest because they represented mostly introduced species that are pantropical or neotropical in distribution.

Only about 5% of the natural vegetation in Puerto Rico is protected; a major Forest Reserve is the well-known El Yunque, managed by the USDA Forest Service, as the Caribbean National Forest. There are about 240 species of trees in this forest of about 1,000 species of higher plants; we sampled 18 of the tree and shrub species and one fern. A preliminary report of our collections (shipping list) was sent to the forester before leaving Puerto Rico. This report indicated species, plant parts and weight of each sample. Additional data on geographical distribution of species and genera sampled, and the number of extracts tested for each genus, are shown in Appendix I. Vouchers were reviewed by Roger Sanders and Guy Nesom at BRIT with minor changes. One species

Left: *Gomidesia lindeniana* (Myrtaceae). Liogier indicates ~40 species in the genus, mostly found in Brazil. Only one extract was listed by the NCI in their RFP 2000 as having been screened in the genus. Right: *Ottoshulzia rhodoxylon* (Icacinaceae), a genus of three species, the one shown here representing an endangered species from which one individual was planted at this site; no samples were collected of this species. According to the US Fish and Wildlife Service, "only nine individuals are known to exist in Puerto Rico" at three locations, the north coast limestone Hills of Bayamon, dry limestone Guanica Commonwealth Forest on the south coast, and "a single individual in the Maricao Commonwealth Forest." Known also from the Dominican Republic where reportedly rare. A species in a related genus, *Nothapodytes foetida*, has been found to contain camptothecin and 9-methoxycamptothecin.

we collected in the Luquillo Forest, *Alchornea latifolia* (Euphorbiaceae), is active from Costa Rica according to a report by the Missouri Botanical Garden with reference to Setzer et al. 2000.[14]

Most of our collections were from the Maricao State Forest Reserve, 43 of 66 species. Here we received additional assistance freely provided by the Chief Forester, Adrian Muniz, that included him, his assistant—Noel Ayala Santana, and two vehicles. Before collecting in this forest, we spent several days conducting surveys of the flora of ~ 845 vascular species of which 278 are trees. The PI was kept very busy identifying ~ 200 specimens collected by the field team (PI, Adrian Muniz, Noel Ayala S., and Richard Marin). The Chief Forester also educated us about the birds in the Maricao forest where we were fortunate to see several rare species such as the Adelaide Warbler.

A shipping list detailing species, plant parts, and quantity collected was provided to the Chief Forester's Office before we left Maricao. From our discussions with Chief Forester Muniz, it would appear that previous collections for the NCI by NYBG did not come from the Maricao Forest. Our samples are represented by three new genera to the NCI screen (*Comocladia, Phialanthus*, and *Siphoneugena*), and we suspect most species are also new, especially those collected on serpentine derived soil such as *Scolosanthus portoricensis* (genus with only 1 extract, Rubiaceae), *Sideroxylon cubense* (35 extracts in genus, Sapotaceae), *Phialanthus grandifolius* (new genus, Rubiaceae) and *Gesneria pedunculosa* (2 extracts in genus, Gesneriaceae).

[14] Setzer, W.N., X. Shen, R.B. Bates, J.R. Burns, K.J. McClure, P. Zhang, D.M. Moriarity, and R.O. Lawton. 2000. *A Phytochemical Investigation of Alchornea latifolia from Monteverde, Costa Rica.* Fitoterapia 71: 195-198. Active against Hep-G2 and A-431 cell tumors.

Left–Coffee dryer at Maricao used to dry plant samples (in the white cotton bags), including plant presses. Right–A team of USDA APHIS Inspectors conducting an inspection of our samples at a shipper's facility near Aguadilla.

Local residents, aware of our collecting for cancer research, reported one plant specifically used for treating cancer. This was identified by the PI as *Petiveria alliacea* (Phytolaccaceae). No samples were collected of this species because of its pantropical distribution. The genus *Petiveria* has only one herbaceous species. The NCI RFP shows that 9 extracts (NCI RFP 2000) that probably were from samples of the entire plant.

Samples were dried in the white collection (cotton) bags by warm dry air that was blown up through the bags resting on a rotating screen used to dry coffee beans. This is similar to the stationary corn dryers we used in Hawaii. Most samples dried within three days.

Before shipping samples, we arranged inspection with APHIS as required. Unlike in Hawaii where we had to take our samples to airport locations, in Puerto Rico, the USDA officials came to the shipping facility to do the inspection. This was not only better for us but also for the USDA since the shipper's facility was better equipped to deal with the fine plant debris that can cause allergies.