Screening for new antitumoral and antibacterial drugs from Brazilian plant extracts

Younes RN*, Varella AD, Suffredini IB

*Extraction Laboratory of the Universidade Paulista – UNIP, and Research Institute of the Hospital Sírio-Libanês, São Paulo, Brazil

Corresponding address to RNY at the Laboratório de Extração Uiversidade Paulista, Av. Paulista, 900, 1 andar, São Paulo, SP, Brazil, 01310-100. Email: extractlab@unip.br

Abstract

Natural products have provided, in the last 40 years, significant number of new drugs currently used in the management of most diseases. The discovery and introduction in the market of important compounds, like paclitaxel, the vinca alkaloids, etoposide, and many antibacterial drugs support the development of programs dedicated to drug discovery. Natural products have been rediscovered as an important tool for drug development despite advances in combinatorial chemistry, due to the complex molecular structures able to interact with mammalian cell targets. The Brazilian flora, the most diverse in the world, has become an interesting spot to prospect for new chemical leads or hits due to its species diversity and associated chemical richness. Screening programs have been established in Brazil as a strategy to identify potentially active substances. High throughput screening techniques allow the analysis of large numbers of extracts in a relatively short period of time, and can be considered one of the most efficient ways of finding new leads from natural products. An updated review of the current status of biological screening program is presented and recent results of new antitumoral and antibacterial chemical leads are discussed.

Key words: biodiversity, screening, natural products, Amazon Rain Forest, Atlantic Forest.

The absolute number of cancer deaths is declining, according to epidemiological studies most developed countries. Nonetheless, cancer still considered a major public health problem, second only to cardiovascular diseases in mortality rates[1]. Systemic chemotherapy for advanced and metastatic disease has evolved dramatically with the introduction of new drugs and regimens, either as isolated treatment, or in adjuvant settings, associated to surgery or radiation therapy[2]. Several new molecules have been developed into commercially available drugs that had origin in extracts derived from natural resources, such as paclitaxel, the vinca alkaloids and etoposide.

Infections are still considered one of the main causes of human and animal morbidity and consequent mortality. Controlling resistant bacteria is an ever-growing endeavor and is a major concern for specialists around the world[3,4,5,6]. The introduction of new antibiotics became a matter of public health. Fortunately, the research in this area is widespread, and, as for cancer, natural products can be considered one of the main sources of new drugs. Data published in 1997[7] and updated in 2003[8] showed that over 50 % of all new antibiotics approved by FDA were extracted from natural sources, or were derived from a natural lead, or synthesized (or semi-synthesized) based on known natural product.

Natural products still play an important role as a source of new antitumoral and antibacterial leads, despite the recent progress in combinatorial chemistry. Although more than 100,000 synthetic compounds can be originated by combinatorial chemistry at any given time, still a high percentage of these products do not present the specific spatial structure required to interact with mammalian targets[9]. The design of new anticancer drugs entered in a new era since then, and combinatorial chemistry is now an important tool for the introduction of new drugs into the market. However, the chemical diversity found in natural products offers new and original options, adding to the thousands of products obtained from combinatorial chemistry. Compounds isolated from plants, especially small molecules, frequently show biological activity as inhibition of macromolecular target, such as proteins. On the other hand, synthetic products, sometimes even a hole library, may not show any significant activity, because most of these products are usually big molecules, with no distinctive biological activity[10].

The effectiveness of drugs such as paclitaxel, docetaxel, etoposide and the vinca alkaloids[11], together with important antibiotics as vancomycin, and penicillin are few examples of the importance of natural products in drug discovery. Moreover, little is known about the pharmacology or the phytochemistry of plants and animals representative of the biodiversity found in countries such as Brazil. The number of superior plants is estimated to be between 200,000 and 250,000 species[12] and only near 20% of the plants have been pharmacologically evaluated[8]. Brazil concentrates 20% of the world's biodiversity[13], and over 17% of the Brazilian biodiversity can be found in the Amazon Rain Forest[14]. The Atlantic Forest contains approximately 35% of the world's Angiospermae, and more than 8% of the Pteridophytae[15]. In view of this species richness and considering that these forests are currently considered as major areas for conservation[16], scientific interest was renewed in Brazilian forests are a potential source for new pharmacological compounds.

Bioprospection of natural resources using screening procedures is not a new technique, for it has been used for decades, but it was implemented in Brazil only recently. The US/NIH National Cancer Institute (NCI) had a large screening program capable of testing 10,000 compounds/extracts per year. More than

114,000 extracts obtained from 35,000 plant species have already been tested in that program, and only 4% have shown significant activity[17]. Nowadays, the NCI bank of extracts has more than 200,000 extracts from marine and terrestrial plant and animal extracts and isolated compounds. From this program, some of the important anticancer drugs were discovered, as paclitaxel (from *Taxus brevifolia* Nutt.), camptothecin (Camptotheca acuminata Decne) and podophyllotoxin/ etoposide (semi-synthetically obtained) (from Podophyllum peltatum L.)[18,19], all of them are currently being routinely used in cancer therapy.

Our group, at the Universidade Paulista-UNIP, in São Paulo, Brazil, concentrates its efforts on collecting plants from the Amazon rain forest (Manaus, AM) and from Atlantic Forest (Iguape, Cananéia e Registro, SP). The university continuously provides the local facilities, including laboratories, boats, and personnel in both regions, as well as a complete infrastructure with capacity for testing approximately 500 extracts a year, in the main laboratory, located in São Paulo.

The establishment of a bank of extracts was a priority, since the beginning of the project. Special attention, investment and technical support have been spent in selecting and processing plant material. For that reason, today the laboratory developed one of the most standardized banks of extracts, composed by plants native to the Amazon and Atlantic Forests. Due to the enormous biovidersity, sample collection strategies had to be defined, in order to create a well established bank of extracts. Plant collection can be based on native traditional knowledge, based on chemotaxonomic information, or on random prospection, i.e., collection of all possible plant samples containing flower or fruit. The random approach is easier in the field, where plants are collected. Special attention is given to plants in the reproductive cycle, allowing for better taxonomic identification. There are advantages and disadvantages in this approach. The advantage is that collecting a good variety of species certainly adds value to the bank of extracts. Conceivably, a wide variety of plants may lead to a wide variety of pharmacological activity and phytochemicals, once the random collection contains both the plants used as medicine by traditional communities and the plants that are not traditionally used, but which may still present active compounds. The downside is the higher investment needed to perform random collections, due to the diversity of natural resources. Effort and significant resources are necessary to process the plants, and more frequent expeditions are needed to detect flowering of the plants on a regular basis. The high number of species to be identified, processed and tested requires a well-established and dedicated technical and scientific personel.

An important issue in natural products drug discovery in Brazil is the regulatory laws that control the access to biodiversity. Bioprospection in Brazil is regulated by strict laws based on the Convention of Biodiversity. The law basically regulates the access to the Brazilian genetic patrimony and allows its bioprospection. That means that any Brazilian citizen or foreigner who desires to bioprospect in Brazil must apply for a license to access the biodiversity and collect material, and another license to do the biological research. Foreigners must have a Brazilian counterpart. Local authorities are trying to establish more effective systems to support the important research efforts developed by established and prospective scientific institutions in the country, allowing more widespread, and at the same time controlled bioprospection. Foreign support to develop meaningful steps involved in finding new drugs is a still necessity, as the Brazilian authorities and private industries do not have the tradition of investing in basic research. Meanwhile, tapping the Brazilian biodiversity is a slow and painstaking endeavor, and the results are slowly showing their potential.

UNIP bank of extracts has approximately 2,000 aqueous and organic extracts obtained from different parts of plants, or from whole plants, depending on biomass availability. Most of them were collected in the Amazon Rain Forest, using a laboraroy boat, especially equiped for the present project.

After collection, the initial plant processing, such as cleaning the material from insects and other species, separating the organs (leaves, stem, fruits, flowers, wood, roots, barks, etc.), is usually conducted inside the boat, as well as the initial taxonomic identifications, up to the level of gender, whenever possible.

The crude plant material is brought to São Paulo to be processed. Plant organs are separated and then completely dried in air-circulating stove at 40 ºC. The material is ground in a hammer-mil. The ground material is placed in glass percolators and an organic extract is obtained through initial maceration with equal volumes of dichloromethane and methanol, followed by maceration with water with the same plant material previously used, resulting in two extracts from each plant. Solvents from organic extract are evaporated with a rotary evaporator and water from aqueous extracts is lyophilized. The organic and aqueous dry extracts are kept in freezers at -20 ºC until use.

Two in vitro biological assays were selected to study the extracts: 1) a cytotoxic assay based on human cancer-cell lines, and 2) a antibacterial assay performed on four resistent bacteria (Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli). As both techniques are conduced in 96-well plates, a large number of samples can be tested in a short period of time, and only a small amount of each sample is required. Because of the small amount of samples needed to run the in vitro assays, only a small amount of each plant organs is collected. This allows

for the collection of a wide range of species on each boat trip into the forests. Recollections of a specific plant are only required whenever extracts show activity in the screening assay, and further identification studies are anticipated.

The antitumoral screening assays performed in the Laboratório de Extração at UNIP is directed against six human cancer cell lines provided by the National Cancer Institute (DTP/NCI/NIH/USA). The cancer cell lines were chosen according to the most prevalent malignant diseases occurring in Brazil[20].The assay is briefly described as follows[21]. Suspensions of breast, prostate, lung, colon, central nervous system, and leukemia cell lines are prepared at concentrations of 10,000, 7,500, 7,500, 15,000, 15,000 and 200,000 cells per well, respectively. After one day incubation, samples of extracts are added at an initial concentration of 100 µg/mL. Microplates are incubated for 48 h before being analyzed by the SRB colorimetric method. Analysis is carried out in microplate reader, at 515 nm. The percentage of lethality is obtained for each sample, and the extracts that are able to kill 15 % of the cell lines are chosen to be fractionated. The fractions are re-evaluated against the cancer cell lines so the active ones are identified and submitted to further fractionation. This procedure continues until the active substances are isolated. The isolated substances are identified using traditional techniques, such as UV, NMR, MS and IR spectrometer analysis, in a collaborative basis. Fractionations are being done now with some of the active extracts.

The antibacterial assay is performed against the above-mentioned four strains of bacteria, obtained from American Type Culture Collection. Briefly, suspensions with defined concentrations of bacteria are prepared and transferred to the microplate wells. The extracts are added to the corresponding wells in a single concentration of 100 mµg/m^{L2}2. After 24-h incubation, the extracts are evaluated and the active ones, i.e., those able to inhibit bacterial growth, are submitted to the analysis of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Extracts showing MIC ≤ 200 mµg/mL, are selected for further bioguided fractionation.

The Laboratório de Extração has now screened over 1,220 plant extracts against the six human cancer cell lines and against the four bacteria, totalizing 12,200 tests. From the initial screening processes, 72 extracts showed significant activity against at least one of the cancer cell lines and 50 extracts showed antibacterial activity against one or more bacteria used in the assay, at the initial concentration of 100 mµg/mL. Those active extracts were fractionated using solid phase extraction syringes and a gradient of solvents composed by dichloromethane, acetonitrile, ethanol, methanol and water. The fractions were tested in the biological assays in a concentration of 100 µg/mL. Results are being currently analyzed and submitted to phytochemical evaluation, in order to further detect the main classes of compounds responsible for the observed biological activity. The compounds within each active fraction should be isolated and identified. Other biological assays should be established in order to evaluate and determine the mechanisms underlying the antitumor or the antibacterial drug activity.

The optimization of drug discovery using screening techniques and high throughput analysis, as well as the advancement of techniques involving spectrometry allowed the identification of tens of thousands active marine and terrestrial natural products, some of them now in clinical trials, as topotecan, irinotecan and camptothecin from terrestrial sources and bryostatin, dolastatin-10 and ecteinascidin 743 from marine sources. Prospecting the Brazilian rain forests is challenging, but the possibility of new effective compounds supports the current efforts in the search for new lead products[23,24,25,26,27,28,29,30].

The encouraging results obtained from the screening project represent an important step towards the effective identification of active drugs against cancer and infectious diseases. Other assays are currently being evaluated and established to evaluate potential activity of the plant extracts against a wide variety of common diseases. Intensive involvement of national and international researchers and laboratories in the systematic screening of active products is required, and will certainly lead to further introduction of more effective drugs against cancer and other diseases into medical armamentarium.

Acknowledgements

The authors thank FAPESP (grant#99/05904-6) and NCI/NIH/USA for the human cancer cell lines.

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