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Molecular bioprospecting of plant extracts: Experience report of the BIOPROS/UFV group in the search for antitumor compounds

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Abstract

This manuscript discusses the experience of the research group BIOPROS (Molecular Bioprospecting in the Sustainable Use of Biodiversity) from the Federal University of Viçosa in the field of bioprospecting. We describe our experience on the search for antitumor compounds from the collection of extracts of native tree species from the Atlantic Forest biome. Presenting an interdisciplinary approach, integrating knowledge of forestry engineering, bioinformatics and natural products chemistry, the bioprospecting research of the BIOPROS group has innovated in the generation of pharmacochemical knowledge of native species of the Atlantic Forest. For the composition of the extract library a total of 220 plant species distributed in 57 botanical families and 140 genera were identified. 196 extracts from 49 plant species were produced; all of them evaluated for cytotoxic activity. By showing the process of obtaining a promising antitumor activity withanolides compounds from *Athenaea velutina*, a species hitherto little known to science, this manuscript shows our sequence of methodological steps used to unravel bioactive natural products from fragments of Atlantic Forest. The research follows the premises of the Convention on Biological Diversity, regarding the creation of strategies for the sustainable use of biodiversity.

Keywords: Extract Library. Pharmaceutical Bioprospecting. Atlantic Forest. Cytotoxic Activity. *Athenaea velutina.* Withanolides.

Introduction

The Convention on Biological Diversity (CBD) established by the United Nations Conference on Environment and Development (UNCED), held in Rio de Janeiro in June 1992, was an important milestone for the international discussion on environment-related issues[1]. Ratified by Brazil in 1998, the CBD established

measures for the identification, conservation, and sustainable use of biological diversity and its components. In this context, research aimed to know the pharmacochemical properties of natural resources are strategic to achieve the objectives of the CBD.

Biodiversity continues to be an important source of new bioactive compounds. The scientific field dedicated to the search for biochemicals, genetic information, and related traditional knowledge from plants, animals, or microorganisms, is known as molecular bioprospecting[2]. In the plant kingdom, different methodological approaches are used to identify natural products, such as ethnopharmacology, chemosystematics, molecular ecology, and computational tools[3]. However, in order to maximize the pharmaceutical bioprospecting, one of the most effective method is to build libraries of extracts from biodiversity[4].

A prominent example of natural resource bioprospecting program was developed by the U.S. National Cancer Institute (NCI-USA), and led to the discovery of important anticancer drugs, such as taxanes and camptothecins[5]. The NCI program also served as a model to other prospecting groups, which resulted in the discovery of potential lead molecules as demonstrated in the works of Fouche et al.[6], Eisenberg et al.[7], He et al.[8], and thousands of others. In Brazil, public universities and research centers have been also screening plants, fungi and marine organisms extracts against tumor cells, pathogenic bacteria, and neglected diseases[9–12].

The large biodiversity found in the Brazilian biomes and ecosystems represents an immense repository of little-known species and its biochemical compounds with high bioactive potential[13]. To highlight, the Atlantic Forest alone hosts about 20,000 plant species, most of them are endemic[14]. The anesthetic coadjuvant Atracurium and the hypertensive Captopril are examples of success medicines derived from lead molecules isolated from Brazilian organisms[15]. However, the release of competitive bioproducts from substances extracted from Brazilian biodiversity is just a promise for the bioeconomy. Among scientists, it is consensus that this scenario could be changed by a cooperative program between research centers, government, and industries[16].

The Molecular Bioprospecting in the Sustainable Use of Biodiversity group (BIOPROS; www.biopros.ufv.br), has been producing extracts from the Atlantic Forest species for many years (BIOPROS Extracts Library). In addition, a computerized platform named MAPA integrates phytochemical and geographic information from the sampled species. Each plant specimen receives ID MAPA number, which is highlighted in stainless steel plates to be fixed in the trees and finally guide the access to genetic heritage through a random strategy. In the laboratory, plant material is processed to extract secondary metabolites by maceration process using solvents with different polarities. The BIOPROS Extract Library have been tested against cancer cell lines by the MTT assay.

Herein, we describe our experience on building the BIOPROS Extract Library in order to prospect new cytotoxic extracts and related compounds. Preliminary results are disclosed reporting the antitumor activity of *Athenaea velutina* (Sendtn.) D'Arcy (Solanaceae), an endemic Brazilian shrub source of withanolide compounds.

We describe our experience on the search for antitumor compounds from the collection of extracts of native tree species from the Atlantic Forest biome. To: inventory a fragment of Atlantic Forest (Mata do Paraíso, Federal University of Viçosa); develop the computerized MAPA platform; fix the ID MAPA plates code on the trees; access leaves and twigs by random strategy; perform plant material extraction; screen extracts against cancer cells; identify natural products responsible for cytotoxic activity.

Materials and Methods

Plant species inventory

We inventoried trees in a fragment of Atlantic Forest known as Mata do Paraíso; a 400 hectares semi-deciduous forest that conserves several native fauna and flora species and belong to Federal University of Viçosa/UFV[17]. All inventoried trees were marked and georeferenced by GPS (*Global Positioning System*). These trees represent the BIOPROS Extract Library *in situ* collection. The voucher specimens were identified (**FIGURE 1**) by specialists and deposited in the VIC Herbarium of UFV, Viçosa, Minas Gerais, Brazil. Species names and botanical families were checked against the database of Brazilian Flora 2020 Project list species[18].

FIGURE 1: Herborization process for making exsiccates deposited in the VIC herbarium of the Federal University of Viçosa.

MAPA Platform

The MAPA computerized platform is software that integrates chemical-pharmaceutical information with geographic location of inventoried plant species of BIOPROS Extract Library *in situ* collection. A unique identification number (ID MAPA) is generated for each specimen taking into consideration the family, genus and species, and following the criteria: a) the first three numbers refer to the family, b) the next three numbers inform the genus, and c) the last three numbers indicate the species. The generated ID MAPAs were highlighted in stainless steel plates and fixed to the trees from Mata do Paraíso forest (**FIGURE 2a**).

Access to biodiversity and plant material processing

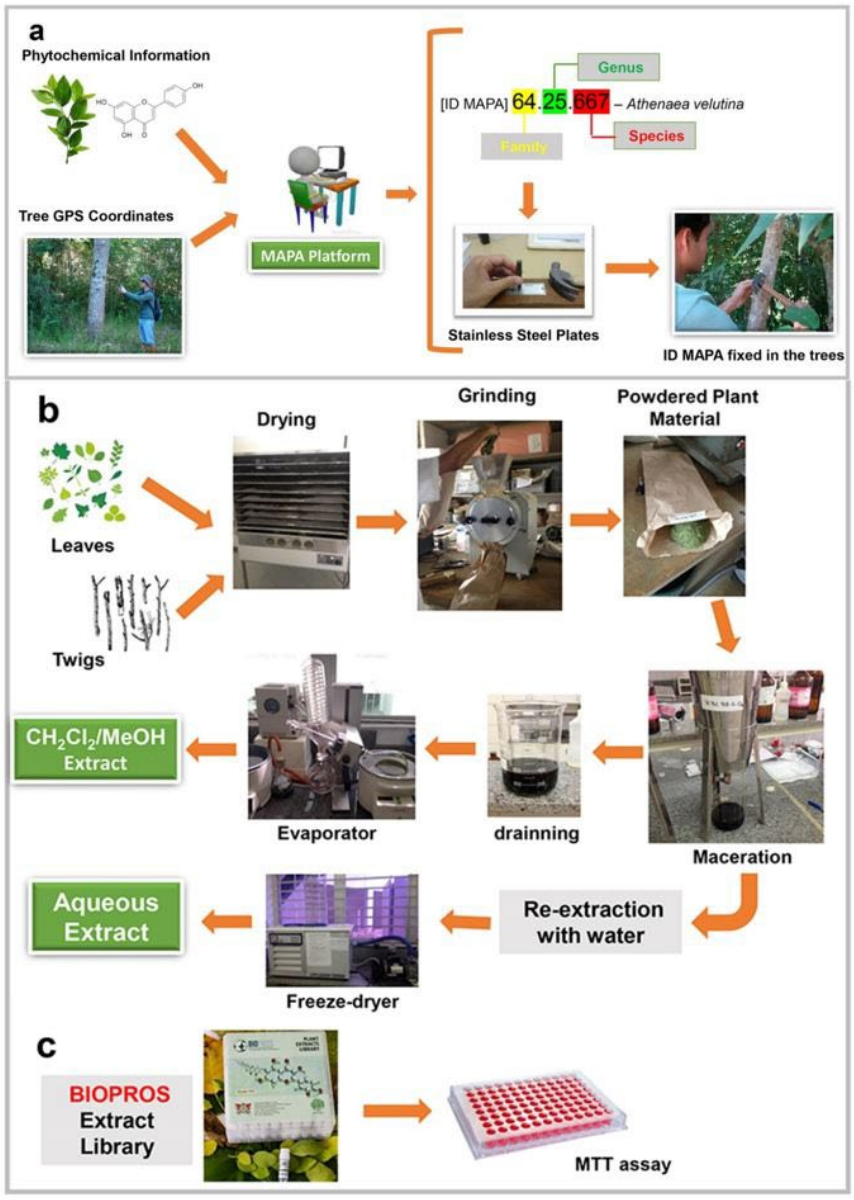
Access to the plant material was authorized by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/number 010134/2014-0), and was always performed using a random strategy. Leaves and twigs were collected with a tree pruner and stored in plastic bags previously identified with the ID MAPA

number. In the lab, the plant materials were cleaned, dried in plant dehydrator (40°C), and grinded in hammer mill (**FIGURE 2b**).

Plant extract production

We used the methodology of plant extracts production adopted by the Natural Products Drug Discovery of U.S. National Cancer Institute[19], adapted to our laboratory. Two extracts were sequentially produced from leaves and twigs of each plant specimen using organic solvent (equivalent mixture of dichloromethane and methanol) and distillated water. The organic liquid extract was drained and concentrated in rotary evaporator to obtain the organic extract. The remaining plant material was re-extracted with distillated water, which is lyophilized resulting in the dry aqueous extract (**FIGURE 2b**).

FIGURE 2: BIOPROS methodology used to search cytotoxic extracts from Atlantic Forest.



Legend: (**a**) The MAPA platform stores phytochemical and geographic information about the species identified in the Mata do Paraíso. This software also generates the ID MAPA code, which is fixed in the trees to guide the plant access. (**b**) The plant material is processed and sequentially extracted with dichloromethane/methanol (1:1) and distillated water. (**c**) The BIOPROS Extracts Library is screened in cancer cell lines.

In vitro cytotoxic test

The cancer cells were grown in culture RPMI medium and maintained in humidified CO_2 (5%) incubator at 37°C. For the assays, the cells were plated in 96-well microplate. The extracts were dissolved in dimethyl sulfoxide (DMSO) and tested at 100 µg/mL. The cytotoxic activity of the extracts was measured by spectrometric absorbance of the formazan reagent (MTT assay) (**FIGURE 2c**). The extracts that reduced the growth of cancer cell lines by 70% (GI_{70}) or more were tested at eight serial dilution concentrations in the range from 200-1.562 µg/mL to determine the IC_{50} (the concentration of the extract that inhibits 50% of cell growth).

Description of the experience

We developed a methodology to bioprospect hundreds of extracts including plant species inventory, rational access to plant material in the forest, extracts manufacturing, and cytotoxic screening.

Via plant inventory, 220 native species distributed in 57 botanical families and 140 genera were identified in the Mata do Paraíso. Nowadays, the BIOPROS Extract Library *in situ* collection is represented by 282 individuals (**FIGURE 3**).

The dichloromethane-methanol (1:1) solvent optimizes the yield of extracts; the time consumed to extraction was reduced draining the solvents once (the extraction time for each type of chemical solvent is 15 h); the reuse of the dichloromethane-methanol solvents (distillated in rotary evaporator) reduced the costs with chemical reagents; the aqueous extracts from leaves and twigs had lower yields and higher time consumption due the lyophilization. The average yield of extracts obtained for each extraction solvent for different parts of the plants, are shown in **TABLE 1**. Furthermore, the use of different solvents produces extracts containing a representation of all molecules found in the specimen[19]; since that organic solvent removes nonpolar and intermediate polar compounds, while water extract contains substances with high polarity.

FIGURE 3: Map of georeferenced trees (*in situ* collection) from the BIOPROS Extracts Library at the Mata do Paraíso forest.

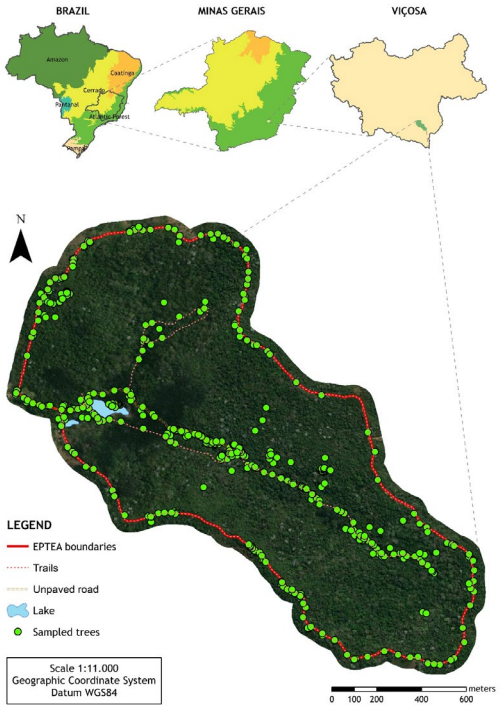


Table 1: Overall average yield (%) and cytotoxic activity (%) of the BIOPROS Extract Library.

Plant Material	Leaves		Twigs	
Extract	CH_2Cl_2/MeOH (1:1)	Aqueous	CH_2Cl_2/MeOH (1:1)	Aqueous
Yield (%)	6.4%	3.3%	3.3%	2.8%
Cytotoxic (%)	2.5%	1.0%	1.5%	0%

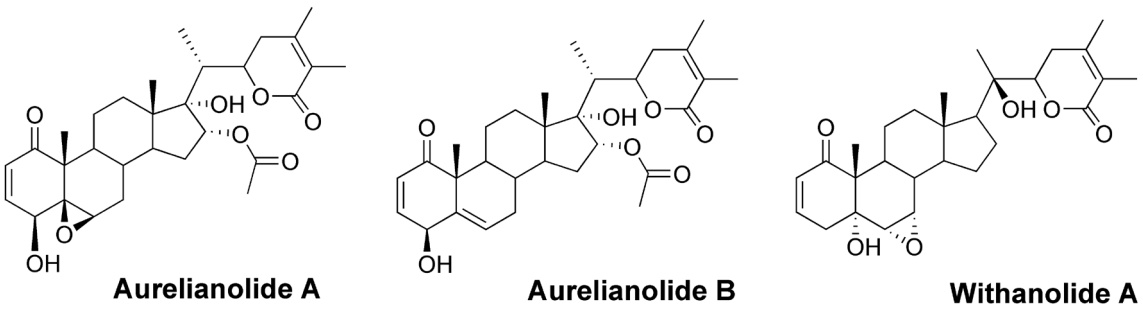
CH_2Cl_2: dichloromethane; MeOH: metanol

We produced 196 extracts from 49 plant species in one year. During a week, for instance, using two maceration equipment, it was possible to produce up to 32 extracts. The cytotoxic assay was also optimized. In each 96-well microplate, 31 extracts and the controls could be tested in triplicate. Our preliminary results reported the cytotoxicity of *Tabernaemontana hystrix, Vernonanthura polyanthes, Croton celtidifolius, Maclura tinctoria, Syzygium jambos, Casearia sylvestris* and *Acnistus arborescens.* Therefore, a hit rate of 14% was obtained based on the number of plant species with cytotoxic activity amongst the 49 investigated species. Overall, the dichloromethane-methanol extracts of leaves were the most cytotoxic followed by the extracts of twigs using the same mixture of solvents (**TABLE 1**).

However, as main results, we disclosed for the first time the cytotoxic potential of *Athenaea velutina*, an endemic plant species from Brazilian Atlantic Forest[20]. Further studies showed that *A. velutina* extract inhibited *in vitro* cells adhesion, invasion, migration and colony formation. A pharmaceutical formulation containing this extract effectively reduced the metastatic lung black nodules of melanoma B16F10-bearing mice[11].

The antitumor effect of *A. velutina* was attributed to the presence of withanolides , a group of steroidal compounds that occur as natural products built in an ergostane skeleton, which are appropriately oxidized to form a lactone ring. Among the withanolides identified in *A. velutina,* we highlight aurelianolide A, aurelianolide B and withanolide A (**FIGURE 4**). These compounds induced cancer cell death by apoptosis and promoted cell cycle arres t[21].

FIGURE 4: Withanolides identified from *A. velutina* leaves. The both aurelianolides A and B were previously isolated in *Athenaea fasciculate*[22].



Results and Discussion

The Convention on Biological Diversity, which is an international agreement that recognizes the sovereign rights of States over their natural resources, gave new dimension for molecular bioprospecting with clearer rules for the sustainable use, conservation, and equitable sharing of benefits derived from biodiversity[1]. It also provided an institutional framework for the promotion of scientific studies of biodiversity between developing countries – high biodiversity holders –, and industrialized nations. Thus, the development of

methodologies for the rational investigation and use of bioactive natural products support the conservation of natural resources[23].

A bioprospecting program based on the sustainable use of life biodiversity is paramount to developing countries such as Brazil, not only to reap economic benefits but also to promote the protection and conservation of biodiversity in accordance with the CBD's guidelines[1]. To reach this purpose, bioprospecting technologies must be developed in order to increase the knowledge about the diversity of life, which could be employed for the identification and use of new biotechnological valuable products to mankind[9,13,16]. It is the aim of the BIOPROS group that has been implemented a large-scale project to produce and test extracts from the Atlantic Forest trees, where species are still rarely studied for their biological activity. The BIOPROS Extract Library is also screened in other biological targets such as antibacterial[24,25], Anticholinesterase inhibitor[26], Insecticidal[27], and phytonematode control[28].

Our molecular bioprospecting approach relies on the accuracy of scientific data on the taxonomic information and localization of organism sources using GPS dates. The traceability of the trees in the forest and the labeled ID MAPA plates is the great feature of our extract library, whereas this enables the supply of more plant material from the same individual, reducing metabolic variations due to edaphoclimatic conditions. In addition, the pharmacological information about the species available in the MAPA database guide the phytochemical studies. An efficient method of extract manufacturing and cytotoxic screening was standardized by us leading to the identification of species with potential pharmacochemical use. Our major discovery was to report the unpublished cytotoxic activity of *Athenaea velutina*. Other studies have been conducted with *A. velutina* leading novel withanolides isolated and toxicological safety proven (unpublished data).

Conclusion

In conclusion, pharmaceutical bioprospecting in plant extracts from the Brazilian Atlantic Forest can be an important factor for the conservation and sustainable use of biodiversity, following the guidelines of the Convention on Biological Diversity. Furthermore, it can contribute to increasing the therapeutic arsenal, aiming at the development of new drugs.

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