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The use of an integrated molecular-, chemical- and biological-based approach for promoting the better use and conservation of medicinal species: A case study of Brazilian quinas

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ABSTRACT

Ethnopharmacological relevance: Quina is a popular name originally attributed to *Cinchona pubescens* Vahl (=*Cinchona succirubra*) and *Cinchona. calisaya* Wedd., species native from Peru that have the antimalarial alkaloid quinine. In Brazil, bitter barks substitutes for the Peruvian species began to be used centuries ago, and they still are sold in popular markets. To assess the authenticity and the conditions on which samples of quinas have been commercialized, using the DNA barcode, chemical and biological assays.

Materials and methods: Starting with 28 samples of barks acquired on a popular market, 23 had their DNA extracted successfully. The regions matK and rbcL were amplified and sequenced for 15 and 23 samples, respectively. Phytochemical analyses were performed by chromatographic methods, and biological essays were done by antimalarial tests in vitro.

Results: The identified species belonged to six different families, many of them endangered or with no correlation with use in traditional medicine as a Brazilian quina. The absence of typical bitter chemical substances indicated that barks have been collected from other species or from very young trees. The results of biological essays confirm the lack of standardization of the sold materials.

Conclusion: The integrated approaches proved to be efficient to evaluate medicinal plants sold in popular markets and can be useful for promoting their better use and conservation.

1. Introduction

According to the World Health Organization (WHO), between 65% and 80% of the population in developing countries currently uses medicinal plants as remedies (WHO, 2011). In Brazil, native species have been used for millennia by the Amerindians, and many important medicines, as pilocarpine, tubocurarine and emetine, were identified from their knowledge (Li and Vederas, 2009; Nogueira et al., 2010). Currently, medicinal plants are still in use by both rural and urban Brazilians, but most of the plants that are used are composed of exotic species, that were introduced to the country during the early phases of European colonization, in the 1500s. This phenomenon is also related to the intermingling of cultures that has occurred during the previous centuries, as well as the result of the continuous destruction of the rich Brazilian ecosystems. In fact, the accelerated destruction of native vegetation has contributed to a gradual loss of native medicinal species and the traditional knowledge about them (Shanley and Luz, 2003; Voeks and Leony, 2004). Today, only 7% of the Atlantic forest is preserved, and other ecosystems, such as the savannas (cerrado) and caatinga, are gradually being replaced by monocultures of eucalyptus, sugarcane, soybeans and livestock (Giulietti et al., 2005; Mittermeier et al., 2005). This situation highlights the necessity to promote the adequate use and conservation of medicinal species from Brazilian biodiversity, especially those used in traditional medicine.

Among the old-used native medicinal plants are those named quina. This name is originally attributed to *Cinchona pubescens* Vahl (=*Cinchona succirubra*) and *Cinchona calisaya* Wedd (Rubiaceae),

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native from Peru and which bark furnishes the bitter alkaloid quinine. Besides it uses to treat malaria, quinine is also currently utilized as a flavor in tonics, bitters, aromatized wines and other spirits (Stewart, 2013). Knowledge of the febrifuge effect of the quina's barks was assimilated from Amerindian culture by the Spanish colonizers in the 17th century (Paz-Soldán, 1941). This remedy was introduced to Europe in 1640 by the Jesuits (named Jesuit's powder), and until the 18th century, the European countries, which at the time were expanding their population in tropical regions, sought to spread the cultivation of *Cinchona*. The Portuguese, Brazil's colonizers, also followed this practice and began growing *Cinchona* in various African colonies (Ferrão, 2005). In Brazil, however, they promoted the search for substitutes of these barks from the native vegetation (Peckolt, 1916; Souza, 1951). As a consequence, several other bitter plants were discovered, which also became referred to as quina, and were used as substitutes for *Cinchona* in Brazilian traditional medicine. In a recent study, we showed data from 29 such species that have been used historically. The study showed that *Remijia ferruginea* (A.St.-Hil.) DC. (Rubiaceae) and *Strychnos pseudoquina* A. St.-Hil. (Loganiaceae) were the most widely used as substitute of the true quina from Peru.

Since 1995, the Brazilian Ministry of Health, following the recommendations of the WHO, started to establish a set of herbal regulations to improve the quality of commercial herbal products. By these regulations, only plants with criteria of efficacy and security can be used for preparing industrialized herbal products. The lack of scientific information that confirms the efficacy and security of native Brazilian species has led, as consequence, their intensive commerce in popular markets. In a previous study, we showed the positive effect on the harvesting of native species by pharmaceutical companies, after the establishment of these regulations (Brandão et al., 2010). The impact of collecting native plants sold in popular markets, however, is more difficult to be estimated because they are obtained in the wild without control or voucher herbarium samples, for accurate identification (Melo et al., 2009).

The use of molecular techniques has been proven to be a powerful tool for the taxonomic identification of medicinal plants, including species with very similar morphological and chemical characteristics (Herbert et al., 2003; He et al., 2010; Gao et al., 2011; Zuo et al., 2011), as well as crude vegetal drugs sold in market (Kool et al., 2012; Newmaster et al., 2013). In this study, we used the molecular identification integrated to chemical and biological methods, which authenticate and verify the conditions in which species named quina have been collected and commercialized in a popular market of Brazil. The final objective is to contribute for the better use of these remedies and their conservation.

2. Materials and methods

2.1. Collection of the commercial samples

Twenty-eight samples of barks marketed with named of "quina" were purchased from the Popular Market of Belo Horizonte, a city of 2,500,000 inhabitants located in the Southwestern region of Brazil in Minas Gerais. The samples were transported to the laboratory at the vegetal drug collection (DATAPLAMT) of the Museum of Natural History and Botanic Garden of Federal University of Minas Gerais (samples Q-1 to Q-28), where they were registered and conditioned in an acclimatized room.

2.2. Reference samples

Barks of *Remijia ferruginea* (A.St.-Hil.) DC. (Rubiaceae) and *Strychnos pseudoquina* A.St.-Hil. (Loganiaceae) were collected in Diamantina (18° 5′51,1″/ 43° 27′51,3″) and Curvelo (18° 49′49,8″/ 44° 32′17,4″), respectively, and used as reference samples. These species were selected for this procedure because they were widely used in the past and considered the official Brazilian quina in Pharmacopoeia (WHO, 2005; Brandão et al., 2008). Voucher specimens were deposited in DATAPLAMT (DAT201-DAT209). Following the standards recommended by the International Consortium for the Barcode of Life (IBOL - www.ibol.org) for DNA Barcode identification, five samples of *Remijia ferruginea* and four from *Strychnos pseudoquina* were used to provide DNA barcode reference vouchers. A total of eighteen sequences were generated and deposited in GenBank (Table 1).

Table 1
Voucher identification and GenBank deposit number of the DNA Barcode sequences for reference samples.

Species	Voucher[a]	GenBank acession	Region	Sequence
Remijia ferruginea	DAT201	KF667963	*matK*	505 pb
		KF667946	*rbcL*	524 pb
Remijia ferruginea	DAT202	KF683518	*matK*	718 pb
		KF683525	*rbcL*	613 pb
Remijia ferruginea	DAT203	KF683519	*matK*	718 pb
		KF683526	*rbcL*	613 pb
Remijia ferruginea	DAT204	KF683520	*matK*	718 pb
		KF683527	*rbcL*	613 pb
Remijia ferruginea	DAT205	KF683521	*matK*	718 pb
		KF683528	*rbcL*	613 pb
Strychnos pseudoquina	DAT206	KF667964	*matK*	505 pb
		KF667947	*rbcL*	524 pb
Strychnos pseudoquina	DAT207	KF683522	*matK*	739 pb
		KF683529	*rbcL*	613 pb
Strychnos pseudoquina	DAT208	KF683523	*matK*	739 pb
		KF683530	*rbcL*	613 pb
Strychnos pseudoquina	DAT209	KF683524	*matK*	739 pb
		KF683531	*rbcL*	613 pb

[a] The Vouchers are deposited in the DATAPLAMT collection – Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais – Brazil.

2.3. DNA extraction

The DNA was extracted from the bark of plants using the DNeasy plant mini kit (QIAGEN – catalog number 69106) with modifications. Approximately 20 mg of each sample was macerated using a mortar at room temperature. The powder was mixed with 600 uL of the buffer AP1 supplied with the kit and incubated at 65 °C and 400 rpm for 1 h in Heatblock (Eppendorf – Thermomixer compact). After that, 230 uL of the buffer AP2 supplied with the kit was added, and the samples were incubated on ice for 30 min. The lysate was centrifuged for 5 min at 20.000 × *g* in microcentrifuge (Eppendorf 5417c Rotor FA-45-24-11). The lysate was pipetted into a QIAshredder Mini spin column with a 2 mL collection tube attached supplied with the kit and centrifuged for 2 min at 20.000 × *g* in microcentrifuge (Eppendorf 5417c Rotor FA-45-24-11). The flow-through was transferred into a new tube, 1.5 volumes of Buffer AP3/E supplied with the kit was added to it and mixed by pipetting. A volume of 650 uL of the mixture was transferred into a DNeasy Mini spin column attached to a 2 mL collection tube supplied with the kit and centrifuged for 1 min at 6000 × *g* in microcentrifuge (Eppendorf 5417c Rotor FA-45-24-11). The flow-through was discarded and this step was repeated with the reminder of the sample. The spin column containing the DNA was attached to a new 2 mL collection tube and 300 ul of buffer AW supplied with the kit was added and centrifuged for 1 min at 6000 × *g* in microcentrifuge (Eppendorf 5417c Rotor FA-45-24-11). This step was repeated two more times to clean the DNA. In the last repetition, the column was centrifuged at 20000 × *g* in microcentrifuge (Eppendorf 5417c Rotor FA-45-24-11) for two minutes to completely dry all of the ethanol. The spin column was transferred to a 1.5 mL microcentrifuge tube and 50 uL of