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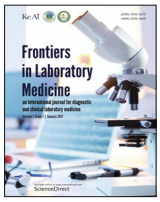

Green extraction of endemic plants to synthesize gold nanoparticles for theranostic applications

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ABSTRACT

We report a simple and original method to synthesize water-soluble gold nanoparticles in which a polyphenolic fraction, extracted by two plants originated from Reunion Island, was mixed to tetrachloroauric acid ($HAuCl_4$) leading to shell-like hybrid flavonoid-metal nanoparticles (NPs). The nanoparticles have been characterized by ultra-violet/visible Raman spectroscopy, and also by electron microscopy imaging (TEM). The results of these analytical green methodologies highlight nanometric sized, stable, hybrid complexes of about 15 nm, or flower-shaped 40 nm diameter with outer surface rich of functional chemical groups. This paper, through an original chemical approach, will occupy an important position in the field of Nanomedicine, and the authors hope that novel perspectives will be proposed for the development of a double nanotheranostic (plasmonic phototherapeutic and X-ray based computed tomography (CT scan)) in order to treat cancer simultaneously by plasmonic phototherapy (PTT), and at the same time to allow the visualization by X-ray based computed tomography.

Introduction

Nanotechnology in health provides challenging environment for the treatment of cancer, especially in drug delivery through nanoparticles in the case of targeted therapy[1,2] The growing interest for inorganic nanoparticles in medical diagnostic and oncology shows the focus of the research area on the new way of treating cancer, rather than the claim of the toxicity of vectorized molecules.[3] Gold nanoparticles have been widely studied due to their unique surface plasmon resonance (SPR) and their applications in biomedical science including drug delivery,[3–6] tissue/tumor imaging and photothermal therapy.[7] Eco-friendly and cost-effective procedures for the synthesis of nanoparticles represent interesting topics for biologists, chemists and materials scientists, especially in light of the efforts to find greener methods of inorganic material synthesis. Reunion Island is a French overseas department emerged from the Indian Ocean about three million years ago. The biodiversity of Reunion Island is endemic at 40% due to its isolation from other lands. The use of plants as traditional medicine is very common among the local people. Thus, these two criteria would also allow to discover many plants with interesting properties. In this work, two plants registered in French pharmacopeia were studied for their antioxidant virtue: *Hubertia ambavilla* and *Hypericum lanceolatum*. Results showed a high concentration of flavonoid molecules in these two plants. *H.ambavilla* is used traditionally for circulation, diabetes, eczema, wounds, lichen tropicus, rheumatism, itch, asthma and ulcer. Phytochemical studies also revealed the presence of flavonoids, tannins, leucoanthocyanes, and phenols.[8] Methanolic extracts of the leaves and stems have shown radical scavenger and antioxidant activities.

H.lanceolatum is arborescent species originated from Reunion Island in Indian Ocean. It is also found in Africa, from Equatorial Guinea to Tanzania. The essential oil of the flowers of this plant is used in phytotherapy. This plant is used in traditional medicine in the form of herbal tea to relieve heartburn, urinary disorders, and relieve and regularize painful menstruation or even fever.

This report herein describes a new way to obtain aqueous polyphenolic extract from these two plants and demonstrates their

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capacity to act as reducing agent in the synthesis of gold nanoparticles. Among these flavonoids, five molecules are described: rutin, quercetin, isoquercetin and hyperoside (Scheme S1 Supporting Information). Their concentration changes in function according to the season and the altitude of the collection.

Quercetin is a flavonol widely used because of its pharmaceutical properties effective against a wide variety of diseases, including cancer. Most of the beneficial effects of quercetin are related to its antioxidant properties which may result from its ability in scavenging free radicals and chelating metal ions.[9] There are several methods to synthesize gold nanoparticles. This synthesis requires, in general, a gold precursor that is reduced in Au^0, then aggregated to form gold nanoparticles (AuNP) and is stopped in its growth by a blocking agent. This latter molecule can also be used to stabilize the resulting AuNP. The reducing agent acts as a stabilizer.

The Turkevich protocol is the oldest and the most commonly used one.[10] It presents a simple and quick way to obtain gold nanoparticles with a diameter comprised between 12 and 20 nm and a correct narrow size distribution (10–16% standard deviation). An aqueous solution of ions tetrachloroaurate ($HAuCl_4$) is reduced by sodium citrate, which has the triple role of reducing agent, blocking agent and stabilizer. Seitz[11] has optimized this protocol by controlling the temperature. Frens[12] also adapted the initial protocol, ten years after Turkevich, to obtain 16 to 147 nm diameter nanoparticles by changing the molar ratio of citrate/Au^{3+} in solution. The decrease in this ratio leads to the increase in the size of the nanoparticles as the gold atoms agglomerate to form a smaller surface area that requires less citrate for stabilization.

This experiment demonstrated the independence of nucleation and growth phenomena of the metal nanoparticles. This method leads to nanoparticles stabilized by weakly bound ligands to gold, which makes their displacement possible by other molecules for subsequent functionalization or grafting, and limits the formation of aggregates in solution. Professor Kattesh Katti pioneered the synthesis of gold nanoparticles by green chemistry.[13] Other teams have synthesized gold nanoparticles from natural products like grapes, roses, honey or lemon grass leaves.[14] This led, most of the time, to spherical AuNP.

No results were reported concerning the synthesis of gold nanoparticles with *H. ambavilla* and *H. lanceolatum*. Furthermore, no results were reported on the type of flavonoids being used and their role during the reduction, nucleation and stabilization of AuNP.

Stylianopoulos[15] reported that it is important to carefully design nanoparticle in order to overcome barriers imposed by the tumor microenvironment, which could result in better treatments. Gold particles of 40–50 nm sizes were able to more effectively bind and induce receptor-mediated endocytic processes. It has been shown that spherical particles less than 100 nm in size are internalized more effectively than rod-shaped and elongated particles. For larger size particles, however, internalization is faster and more efficient for elongated particles (high aspect ratio). Spherical nanoparticles move with the flow and elongated particles can rotate which permits a closer approach to the vessel wall. The leakiness of the vessels near the tumor facilitates the penetration of the nanoparticles because of bigger size pores (10 nm to 2 μm).

Two endemic plants originated from Reunion Island and registered in the French pharmacopeia were used to synthesize gold nanoparticles: *H. ambavilla* (leaves) and *H. lanceolatum* (flowering tops). Two original methods were developed to extract bioreducing agents used to synthesize gold nanoparticles, in substitution to citrate which is mostly used in the conventional methods.

- The first protocol was a crude extraction performed for the two plants, in order to obtain the concentrate of reactive species that could reduce Au^{3+} in Au^0 and stabilize the newly-formed AuNP.
- The second protocol consisted in isolating the fraction containing the concentrate of flavonoids, which are known to have an antioxidant activity

We envision that this study will occupy an important position in the field of Nanomedicine, and hope that novel perspectives will be proposed for the development of nanotheranostics. To the best of our knowledge, these aspects have never been described before in published reports.

Materials and methods

Tetrachloroauric acid ($HAuCl_4$), water milliQ, ethanol (98%) and Acidic aqueous Solution (pH 4) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Extract Plants were purchased by Reunion Ile.

Preparation of the plant extracts and their quantification

Isolation method to obtain the crude polyphenolic extract

Plants were freshly collected and then washed with deionised water. Three grams of material was mixed with 50 mL of deionized water and then heated at 60 °C for 5 min, allowing the release of biological matrices by the rupture of the plant cells. The clear supernatant was cooled to room temperature, and then cooled with ice for 10 min. The filtration was made with a sintered porosity 2. We obtained a brown solution with *H. lanceolatum* and a green solution with *H. ambavilla.* No organic solvent was used during this extraction.

Isolation method to obtain the concentrate of flavonoids plant fraction

We managed to isolate the only fraction containing the concentrate of flavonoids. Cold maceration was used as the extraction technique. The plants were crushed (pore diameter of 10 mm sieve) and then macerated under stirring (150 rpm) for 20 h at room temperature. A 50:50 *milli-Q*™ water and ethanol in solid-solvent ratio of 1:20 was chosen for the extraction of the active ingredients on the basis of the results obtained by Cujic et al.[16] which may reach a better yield regarding the phenolic compounds. After extraction, the macerated material was filtered and reduced to dryness under reduced pressure (temperature maximum bath: 45 °C, Pressure: 150 to 50 bar) and then lyophilized (48 h). The extraction of *H. ambavilla* under these experimental conditions reached a yield close to 50%.

Antioxydant measurements of polyphenolic fractions

- Spectroscopic assays

Content in phenolic compounds (test FOLIN). A method of spectroscopic assay was used (Folin test) to assess the phenolic content of the crude extracts of each plant. The Folin assay (Folin–Ciocalteu) permits the measurement of the content of phenolic compounds in a sample. In basic solution, the Folin–Ciocalteu reagent ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) which are initially yellow can react with the oxidizable groups of polyphenolic compounds present in the plant extract and is reduced to a mixture of blue metal oxides (W_8O_{23} and Mo_8O_{23}). This color is measured by reading the absorbance at 765 nm.

It is proportional to the amount of polyphenols present in the analyzed sample.