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Potential of Application of Natural Products Nanoparticles in Hypercholesterolemia †

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Abstract: Hypercholesterolemia is a condition characterized by high blood cholesterol levels. Currently, the drugs available on the market to treat hypercholesterolemia show adverse side effects, related to their chronic use. Consequently, there is an increased search for alternatives and complementary strategies, specifically supplements based on natural products. Moreover, various studies suggest that supplements with high antioxidant activity have a vital role in the prevention of various diseases. In this work, an aqueous avocado extract was prepared and characterized, to assess its potential in hypercholesterolemia. Also, a delivery nanosystem using albumin nanoparticles was developed to use this extract as a food supplement.

Keywords: natural products; hypercholesterolemia; nanoparticles; *Persea americana*

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1. Introduction

Hypercholesterolemia, one of the main risk factors of cardiovascular disease (CVDs), is characterized by high plasma cholesterol levels [1,2]. The available treatment for this condition consists of a combination of two types of approaches, one regarding the individual's lifestyle changes and the other concerning drug therapy. Among the drugs available on the market, the two most frequently prescribed are statins and ezetimibe [3]. Regarding statins, these drugs act by binding to the active site of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, inhibiting the enzyme responsible for the rate limiting step in cholesterol biosynthesis [4]. In the case of ezetimibe, this drug inhibits the Niemann-Pick C1-Like 1 transmembrane protein. This protein is localized in the apical membrane of enterocytes, the intestinal lining of epithelial cells, thereby reducing cholesterol absorption and uptake [5]. Despite the benefits of these drugs, some individuals have reported side effects associated to their chronic use [6,7]. As a result, the search for alternatives has increased, especially in the market of natural products and food supplements [3].

Natural products are an excellent source of bioactive compounds. Some of them are rich in phenolic compounds, which have a high antioxidant activity, being very promising for health benefits and prevention of several diseases, including CVDs [8,9]. In particular, extracts made of *Persea americana*, commonly known as avocado, have demonstrated the ability to reduce blood cholesterol levels [10,11].

To protect and correctly deliver bioactive compounds, nanoparticles (NPs) are considered a good strategy. NPs, whose dimensions are in the range of 10 to 1000 nm, are solid drug carriers that improve the solubility, stability, and bioavailability of the bioactive compounds. These also provide controlled release of bioactive compounds and, therefore, are widely used in the development of this type of food supplement [12].

The aim of the present study was to prepare and characterize an extract obtained from peels of *P. americana*. Subsequently, the extract was evaluated regarding its potential of application in hypercholesterolemia. To enhance the biological activity and to stabilize its bioactive compounds, the extract was encapsulated with bovine serum albumin (BSA) NPs. The characterization of the resultant nanocarrier is presented here.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Acetonitrile and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from VWR (Radnor, USA). Trifluoroacetic acid was obtained from PanReac (Barcelona, Spain). Bovine serum albumin (BSA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Barcelona, Spain). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640) and Fetal bovine serum (FBS) were acquired from Biowest (Nuaillé, France). Antimycotic and L-glutamine were purchased from Corning (New York, USA). Ethanol absolute anhydrous was purchased from CARLO ERBA (Cornaredo, Italy). Glucose was acquired from HiMedia Laboratory (Mumbai, India).

2.1.2. Cell Line and Cell Culture

A human hepatoma cell line (HepG2) (ECACC 85011430) and human colon carcinoma cell line (Caco-2) (ECACC 86010202) were acquired from *European Collection of Authenticated Cell Cultures*.

2.2. Methods

2.2.1. Extract

The extract under study was obtained from the peels of *P. americana* of the Hass type. This extract was prepared through a 10 min decoction in water of 100 g peels/L. Then the suspension was filtered, frozen and freeze-dried. The resultant extract was stored at −20 °C.

2.2.2. Extract Characterization by High-Performance Liquid Chromatography

The extract was characterized through high-performance liquid chromatographic accoupled to a diode-array detector (HPLC-DAD). For the analysis an Elite LaChrom® VWR Hitachi liquid chromatograph (Tokyo, Japan) equipped with a Column oven L-2300, a Diode array detector L-2455 (VWR, USA) and a Column LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 μm) was used. The separation was carried out for 40 min using a gradient composed of acetonitrile (solution A) and 0.05% trifluoroacetic acid (solution B), as follows: 0 min, 10 % A, 90 % B; 30 min 50 % A, 50 % B; 35 min, 50 % A, 50 % B; 38 min, 10 % A, 90 % B; 40 min, 10 % A, 90 % B. The flow rate was 0.8 mL/min and 25 μL of an extract solution in ultrapure water (1 mg/mL) were analyzed. The detection was carried out between 200 and 600 nm. Calibration curves of chlorogenic acid, catechin and epicatechin were performed in the same conditions.

2.2.3. Determination of Antioxidant Activity of the Extract

The antioxidant activity (AA) of the extract was determined through the 2,2-diphenyl-1-picrilhidrazil (DPPH) method, following the procedure described by Coelho et al.

[13]. The EC_{50}, which is the concentration of the extract showing 50% of DPPH-free radical scavenging activity, was determined. The assays were performed in triplicate.

2.2.4. Safety Assays of the Extract in Human Cell Lines

Caco-2 and HepG2 were cultured in T75 flasks with DMEM and RPMI medium, respectively, supplemented with 10% of FBS, 100 U/mL penicillin, 100 U/mL streptomycin and 2mM of L-glutamine. The flasks were kept at 37 °C in an atmosphere with 5% CO_2. The medium was changed every 48–72 h.

The cytotoxicity of the extract was performed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method, described by Mosmann [14]. The cell viability was evaluated after 24 h incubation, with 100 μL solutions of the extracts in growth medium at different concentrations (0.01–3.0 mg/mL).

A dose-response curve was obtained using a non-linear regression model through *GraphPad Prism 5 software*, where cytotoxicity was plotted as a function of the logarithm of the extract concentration. From this curve, the IC_{50}, which corresponds to the concentration of the extract that leads to 50% cell death, was determined.

2.2.5. Preparation of Empty and Extract-Loaded BSA NPs

BSA NPs were prepared following the procedure described by Santos-Rebelo et al. [15], with some modifications. Firstly, the BSA was dissolved in ultrapure water. Next, the pH was adjusted to 9 (Metrohm© 744 pH Meter, Barendrecht, The Netherlands). Then, this solution was added drop by drop to absolute ethanol under magnetic stirring (Heidolph MR3001, Heidolph Instruments, Schwabach, Germany). Lastly, glucose was added, and this solution was under magnetic stirring for 30 min. The extract-loaded NPs were formulated following the same procedure but by adding extract (5, 10 and 15 mg) to the BSA solution before adjusting the pH. The BSA NPs formulated were stored at −4 °C.

2.2.6. Physicochemical Characterization of BSA NPs

All BSA NPs prepared were characterized in terms of average particle size and polydispersity index through dynamic light scattering (DLS) (Zetasizer Nano S, Malvern Instruments, Malvern, UK) These NPs were also characterized in terms of zeta potential using the electrophoretic mobility technique with the same equipment. For these determinations, all samples were diluted with ultrapure water, where extract-loaded NPs were diluted at 2:10 (*v*/*v*) and empty NPs were diluted at 3:20 (*v*/*v*). The assays were performed in triplicate.

2.2.7. Encapsulation Efficiency of Extract in BSA NPs

The encapsulation efficiency (EE) of the extract in BSA NPs, was determined by an adapting of the procedure described by Pinto et al. [16].

3. Results

3.1. Extract Characterization by HPLC-DAD

A solution of the avocado extract, prepared in ultrapure water (1 mg/mL), was characterized through HPLC-DAD, and the chromatogram obtained is presented in Figure 1.

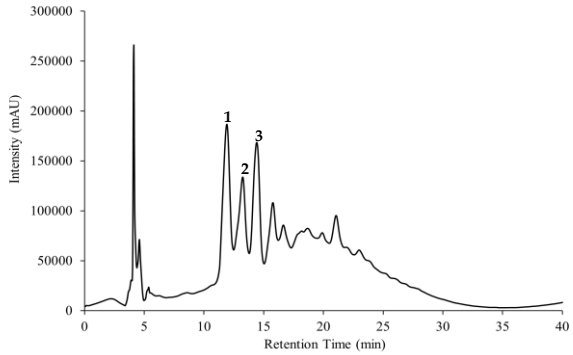

Figure 1. Chromatogram of the avocado extract (1 mg/mL) obtained through HPLC-DAD (250–600 nm): (**1**) Chlorogenic acid; (**2**) Catechin; (**3**) Epicatechin.

By performing calibration curves with several phenolic compounds, three phenolic compounds were identified in the extract, namely chlorogenic acid (1), catechin (2) and epicatechin (3). Chlorogenic acid (1) was quantified as 18.60 ± 0.01 mg/g of dry extract. Catechin (2) and epicatechin (3) were the major phenolic compounds quantified, 73.24 ± 0.01 and 83.14 ± 0.02 mg/g of dry extract, respectively. These compounds have been previously reported as constituents of *P. americana*, specifically, epicatechin was determined as one of the major compounds quantified [17].

3.2. Determination of Antioxidant Activity of the Extract

The antioxidant activity of the extract was assessed through the DPPH method and the EC_{50}, was determined as 6.0 ± 0.2 μg/mL.

Butylhydroxytoluene (BHT) is a synthetic compound that serves as a food preservative and as an antioxidant, commonly used as a standard [17]. This compound was reported to have an EC_{50} of 15.7 ± 0.2 μg/mL [17], indicating that the extract has stronger AA. In a comparison between the EC_{50} value obtained from the literature for an avocado peel extract, EC_{50} = 72.6 ± 10.7 μg/mL [18], with the results of the present study, the extract under study demonstrated a higher antioxidant activity. This performance of the extract can be explained by the higher concentration of phenolic compounds present in the extract relative to the literature [18].

3.3. Safety Assays of the Extract in Human Cell Lines

The safety of the extract was assessed in Caco-2 and HepG2 through the MTT. A dose-response curve for the extract after 24 h of exposure with both cell lines are presented in Figure 2. The toxicity was seen to be higher for HepG2, when compared to Caco-2. However, the extract was not considered toxic since the IC_{50} values obtained for Caco-2 and HepG2, 0.240 and 0.164 mg/mL, respectively, were below 0.100 mg/mL [19]. The low toxicity was also evident from the less accentuated slope of the dose-response curves obtained.

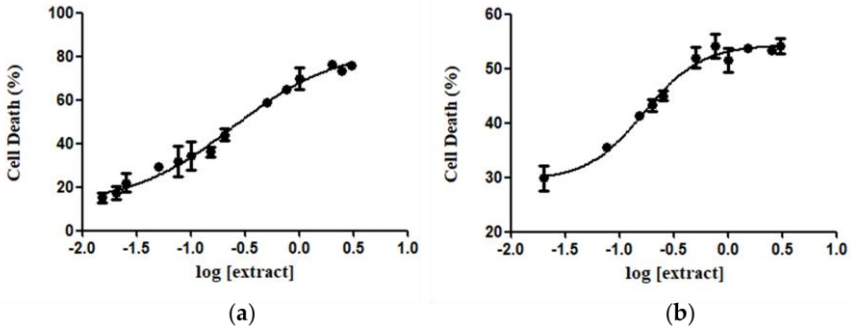

Figure 2. Cytotoxicity of the extract in two cell lines: **(a)** Caco-2; **(b)** HepG2.

3.4. Characterization of BSA NPs

The results obtained from the characterization of BSA NPs, through DLS and HPLC-DAD are presented in Table 1. It was observed that an increase in the quantity of loaded extract resulted in an increase in the size of the NPs. Also, for all of the obtained NPs the PdI was inferior to 0.2 and the zeta potential was negative.

Regarding the EE, BSA NPs with 5 mg of extract encapsulated presented a higher value and also, less extract was unused during the formulation of these NPs. As a result, these NPs were the selected for the next studies.

Table 1. Mean size, polydispersity index (PdI), zeta potential and encapsulation efficiency (EE) of empty NPs and extract-loaded BSA NPs with different quantities (5, 10 and 15 mg). The data are presented as mean value ± SD.

BSA NPs	Mean Size (nm)	Pdi	Zeta Potential (mV)	EE (%)
Empty	220 ± 10	0.151 ± 0.023	-24 ± 4	-
5 mg of extract	275 ± 20	0.170 ± 0.009	-25 ± 7	84 ± 5
10 mg of extract	351 ± 26	0.135 ± 0.044	-30 ± 13	81 ± 1
15 mg of extract	401 ± 5	0.132 ± 0.001	-36 ± 1	77 ± 4

4. Discussion

In the present study, an extract made from *P. americana* peels was evaluated for its potential use as a complementary strategy in the management of hypercholesterolemia and prevention of CVDs. On an initial analysis, HPLC-DAD confirmed that the prepared extract contained important bioactive compounds, such as the phenolic compounds chlorogenic acid, catechin and epicatechin. These compounds have been extensively investigated and many studies have demonstrated that these phenolic compounds can interfere with cholesterol homeostasis, potentially reducing cholesterol levels and improving cardiovascular health [10]. Moreover, this extract demonstrated strong antioxidant activity. This is an important feature to reduce/inhibit free radicals that cause oxidative stress, which are associated with many diseases including CVDs [20]. The high antioxidant capacity of the extract strengthens its potential as a preventive agent for CVDs and various conditions. Regarding the safety assays, the extract revealed no toxicity, which is also a promising result for the development of food supplements.

However, in order to use the extract to incorporate a supplement, it is important to ensure a correctly delivered of an effective dosage. In nanomedicine, it is important that BSA NPs present a small size for oral delivery and a PdI lower than 0.2 to have uniform particles [21]. Also, NPs with a size lower than 300 nm can prevent uptake of substances by enterocytes, cells of the intestine [22]. This suggests that the extract associated to BSA NPs may potentially interfere with cholesterol absorption. Encapsulation results showed that BSA NPs loaded with 5 mg of the extract exhibited the highest encapsulation efficiency (EE). Due to this superior performance, this formulation was selected for further studies. The encapsulation process ensures the stability and controlled release of the bioactive compounds. These preliminary findings suggest that the extract loaded BSA NP may represent a promising delivery system for oral administration of the extract as a potential strategy against hypercholesterolemia.

5. Conclusions

Over the past few years, natural products supplements market has grown due to their health benefits and improvements that consumers are demanding. This study provides stimulating evidence about a phenolic-rich avocado peel extract, encapsulated in BSA NPs, which may offer a novel and effective approach to lowering cholesterol levels and reduce the risk of CVDs. Further research is required to optimize the dosage and delivery

mechanisms, but these preliminary findings represent a promising step towards developing a functional food supplement. As such, cholesterol permeability assays will be carried out in the future, to evaluate the capability of the extract and extract-loaded BSA NPs to reduce cholesterol absorption.

Author Contributions:

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