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"Green"-synthesized zinc oxide nanoparticles and plant extracts: A comparison between synthesis processes and antihyperglycemic activity

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ABSTRACT

Zinc oxide nanoparticles (ZnONPs) have shown antidiabetic activity in multiple studies and can be produced by different plant-mediated ("green") methods. This study aimed to compare ZnONPs prepared via different "green" approaches (heating at high temperatures (400 °C) *vs.* low temperature (70 °C)). The low temperature method involved addition of suspending agents (Tween 80 or gum arabic) and pH variations followed by lyophilization. The study evaluated the hypoglycemic potential of ZnONPs with the best properties (quantity of capped agents and stability) compared to the plant extract *per se*. The ZnONP synthesis involved a mixture of zinc nitrate hexahydrate as the zinc precursor and a plant extract with high antioxidant activity as the capping agent supplier. The results of the studies showed that the procedure using high-temperature heating resulted in almost uncapped nanoparticles with phytocompounds (0.01 % of phenolic compounds) and nanoparticle sizes larger than 300 nm. The low-temperature method produced ZnONPs with high retention of capping agents (92.90 % of phenolic compounds) and a size of approximately 200 nm. The use of Tween 80 with pH adjustment between 9 and 10 resulted in more stable nanoparticles than with gum arabic. These nanoparticles prepared with Tween 80, exhibited a pronounced *in vivo* antihyperglycemic activity at a much lower dose (10 mg ZnO/kg capped by 0.31 mg phenolic compounds per kg) than the extracts alone (400 mg extract/kg) following an oral glucose tolerance test. These results demonstrated that green-synthesized ZnONPs with a high retention rate of phytochemicals can induce antihyperglycemic effects at a low dose.

1. Introduction

According to the International Diabetes Federation's recent Atlas report, diabetes prevalence is rising and is expected to reach 12 % of adults aged 20–79 years old by 2045 (International Diabetes Federation, 2021). Within this figure, type 2 diabetes mellitus (T2DM), also called insulin-resistant diabetes, is dominant and accounts for approximately 90 %. T2DM management is complex and based on a healthy diet, physical exercise and medication to achieve lower glycemia. However, despite treatment, blood glucose levels remain high. Hyperglycemia gradually leads to serious complications over time (Vermeire et al., 2005), which are induced by the increase in reactive oxygen species (ROS) (Kaneto et al., 2010). Many strategies are being studied as alternatives to current treatment for T2DM to improve the effectiveness of medication, including nanoparticulate systems (Nie et al., 2020; Uppal et al., 2018). Zinc oxide nanoparticles (ZnONPs) have attracted

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attention due to the multiple functions of zinc in the management of diabetes. Zinc is known to be involved in the synthesis, storage and release of insulin, the increase in antioxidant enzyme activity, the decrease in inflammation, the decrease in glucose intestinal absorption, the increase in glucose homeostasis and the increase in hepatocyte activity (Farooq et al., 2020; Olechnowicz et al., 2018; Rehana et al., 2017). ZnONPs are produced by different methods, including physical, chemical and biological methods (Droepenu et al., 2022; Naveed Ul Haq et al., 2017). However, physical and chemical methods present some drawbacks, such as the requirement of high energy, high costs, time-consuming operations and the use of toxic chemical reagents that can exhibit adverse effects (Basnet et al., 2018; Rehana et al., 2017). Biological methods can involve the use of plant extracts, algae and bacteria (Naveed Ul Haq et al., 2017). The use of plant extracts (i.e., "green") is simple, involve fewer steps, have a lower cost and is especially interesting because plants are easily available. The bioreducing, antioxidant or antidiabetic phytocompounds they contain (e.g., phenolics) trigger the synthesis of ZnONPs, bind to the surface of the synthesized nanoparticles and can contribute to their activity (Bala et al., 2014; Javed et al., 2020; Tang, 2019), reducing the ROS and thus the damage caused by hyperglycemia. Since antidiabetic phytocompounds are good antioxidants, reducers and capping agents, there is a high probability to synthesize more effective anti-diabetic ZnONPs by choosing plants with high contents of antioxidants, reducing and antidiabetic phytochemicals (Basnet et al., 2018). However, the preparation procedure using plant extracts sometimes requires heating at high temperatures, the addition of suspending agents or pH variations, compromising the integrity of the phytocompounds (Bala et al., 2014; Zhu et al., 2021). Since previous studies have already reported that capping agents contribute to the activity and stability of synthesized nanoparticles (Javed et al., 2020; Rónavári et al., 2017), the study of the presence of phytocompounds and the stability of synthesized nanoparticles is of utmost importance. We have chosen to use stem bark extracts of *Panda oleosa* Pierre among the potential suppliers of active capping agents due to their use in traditional Congolese medicine as an antidiabetic and antioxidant plant (Katemo et al., 2018; Muhoya et al., 2017). Their antioxidant effect was also evaluated. To our knowledge, this represents the first attempt to prepare metallic nanoparticles using this plant extract. We hypothesized that the synthesis procedure (the use of different suspending agents) could affect the product obtained, and to our knowledge, the quantification of capped phytochemicals and the study of the stability of green synthesized zinc oxide nanoparticles in biomimetic media has not yet been performed. This work compared ZnONPs prepared via "green" pathways to the plant extract *per se* by evaluating their hypoglycemic potential.

2. Materials and methods

2.1. Materials

2.1.1. Plants

Herbal material from antidiabetic medicinal plants was used in this study. The leaves of *Azadirachta indica* A Juuss were harvested in August 2020 in Maluku Township (Kinshasa, DR Congo). The leaves of *Moringa oleifera* Lam and *Vernonia amygdalina* (*Gymnathemum amygdalinum* (Delile) Sch.Bip.exWalp) were collected in August 2020 in Lemba Township (Kinshasa, Democratic Republic of the Congo). The stem bark of *Panda oleosa* Pierre was from the Masako reserve (located 15 km EAST-North from Kisangani in the DR Congo) and was collected in August 2020. Their identities were certified under references EKK002082020, EKK003082020, EKK004082020, and EKK001072020 by Mr. Nlandu Lukebiako, a botanist at the Institut National d'Etudes et des Recherches en Agronomie (INERA) of the University of Kinshasa (DR Congo). *Alium cepa* (white and red onions) was bought at the Kinshasa market in July 2020.

2.1.2. Chemicals

Ethanol 99 %, ascorbic acid, methanol and ethylene diamine tetraacetic (EDTA) disodium salt 0.1 mol/L in aqueous solution were provided by VWR International (Leuven, Belgium). Dichloromethane (DCM) was obtained from Merck KGaA (Darmstadt, Germany). Zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$), Tween® 80 (polysorbate 80), sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), pyrogallol and hexamethylenetetramine were purchased from Sigma − Aldrich (St. Louis, MO, USA). Gum arabic and sodium carbonate were supplied by Acros Organics, Belgium. Acetic acid was purchased from Carl Roth GmbH (Germany). Any other chemicals were of analytic grade.

2.2. Preparation of plant extracts

The leaves and stem bark of collected plants were air-dried over one week at room temperature in the dark and then stored away from moisture. Before use, all the dried plant materials were ground using a lab grinder and sieved using a sieve with a pore size ≤ 710 μm to obtain a uniform size range. The bulbs of *Alium cepa* were crushed using a blender just before use.

The preparation of the plant extracts was performed both by decoction (with water as solvent) and by maceration (with 99 % ethanol and with a solution of ethanol 99 %-water in the ratio of 50:50).

2.2.1. Decoction

Fifty grams of ground plant material (or 500 g of fresh onion equivalent to 50 g of dry weight) were heated in 0.5 L of deionized water at 100 °C for 15 min, cooled at room temperature and filtered through Whatman 1 filter paper (Whatman International Ltd, Maidstone, UK). The filtrates were frozen and freeze-dried for 48 h.

2.2.2. Maceration

(i). Fifty grams of ground plant (or 500 g of fresh onion equivalent to 50 g of dry weight) were macerated under agitation for 24 h with 500 mL of ethanol 99 % and then filtered with Whatman 1 filter paper. After filtration, the residues were recovered and macerated again in 300 mL of solvent for 24 h. Then, they were filtered and macerated once again for 24 h in 300 mL of solvent. The filtrates were combined, and ethanol was evaporated first with a rotavapor and then under a laminar flow hood for 48 h.

(ii). By using a mixture of ethanol 99 % and water in a ratio of 50:50, 50 g of ground plant (or the equivalent of fresh onion) was macerated under agitation for 24 h with 500 mL of ethanol 99 %:water (50:50) and then filtered with Whatman 1 filter paper. After filtration, the residues were recovered and macerated again in 300 mL of solvent for 24 h and then filtered and macerated once again for 24 h in 300 mL of solvent. The filtrates were combined, and ethanol was first evaporated on a rotavapor. The filtrates were then frozen and freeze-dried for 48 h.

2.3. Selection of the plant extract

To select the plant extract for the synthesis of nanoparticles coated with radical scavenging compounds, the antioxidant activity of each extract was evaluated by analyzing their DPPH scavenging effect (% reduction) following the method used by Tohidi et al. (Tohidi et al., 2017) with slight modifications. Briefly, 20 μL of a 500, 1000 and 2000 μg/mL solution of each extract dissolved in methanol was added to 180 μL of 0.5 mM DPPH solution in methanol to obtain 50, 100 and 200 μg/mL as final concentrations of the plant extract per well (96-well plates). The mixtures were shaken and allowed to stand at room temperature for 30 min. Controls including methanol (blank) and a DPPH solution (control) were also prepared. Then, the absorbances were measured at 517 nm using a UV–VIS spectrophotometer (Spectramax® ID5, Molecular Devices LLC, USA). Methanolic solutions of ascorbic acid (50, 100 and 200 μg/mL) were used as references for free radical scavenging activity. Lower absorbance values of the reaction mixture indicated

higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the equation (1):

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A0 - A1}{A0} X100 \quad (1)$$

where A_0 is the absorbance of the control (DPPH solution), and A_1 is the absorbance in the presence of all of the extract samples and reference. All tests were performed in triplicate and expressed as the mean ± standard error of the mean (SEM).

2.4. Soxhlet extraction

From the plant whose extracts were found to be more antioxidant and therefore rich in phenolic compounds, a second extraction was performed using dichloromethane to test the antihyperglycemic activity. Dichloromethane is an apolar solvent and has shown good capacity to extract phenolic phytocompounds (Alara et al., 2021; Damilola et al., 2021; Rinthong and Maneechai, 2018).

In brief, on the one hand, 50 g of the plant material of the most antioxidant plant was loaded into the Soxhlet apparatus and allowed to undergo dichloromethanic extraction (250 mL) for 8 h. On the other hand, 2 g of the aqueous extract from the most antioxidant plant was loaded into a Soxhlet apparatus and allowed to undergo dichloromethanic extraction (50 mL) for 8 h. The dichloromethanic extracts were concentrated separately by solvent evaporation using a rotavapor and a hood and then stored at 4 °C until further use.

2.5. Green synthesis of zinc oxide nanoparticles

Green synthesis was performed using the aqueous extract (more commonly used) with the highest antioxidant effect via two procedures. In accordance with the literature (Rehana et al., 2017), the first procedure consisted of mixing zinc nitrate hexahydrate with an aqueous extract solution at room temperature under stirring and subsequently increasing the temperature to 70 °C. Different reactant ratios and reaction durations were used and are described in Table 1. As described in previous works (Doan Thi et al., 2020; Rehana et al., 2017), the mixture was then heated at 400 °C (high temperature method) for 1 h to obtain ZnONPs.

The second method was based on mixing zinc nitrate hexahydrate with the selected plant extract solution at room temperature while adding Tween 80 (3.5 % v/v) or gum arabic (1 % w/v) under stirring (to avoid the growth of large particles). The pH was adjusted to values between 9 and 11 (as described in Table 2 below) by adding a 2 M sodium hydroxide solution dropwise. Then, the mixture was heated at 70 °C under stirring for 1 h and centrifuged (10,000 rpm for 10 min) to obtain the precipitate of ZnONPs (Naseer et al., 2020). The resulting nanoparticles were washed twice with Milli-Q water and lyophilized (low-temperature method).

2.6. Characterization of the synthesized ZnONPs and quantification of capped phytocompounds

Throughout the different processes, an aliquot of the synthesized nanoparticles from each method was suspended in water at a concentration of 0.04 % w/v and was then characterized by UV–visible spectrophotometry using a NanoDrop™ 2000 (Thermo Scientific/USA) and dynamic light scattering (DLS) using a Malvern Zetasizer Ultra instrument (Malvern Panalytical Ltd/UK). For UV–visible spectrophotometry, a drop of this suspension was deposited onto the lower measurement pedestal of the NanoDrop™ 2000, and then, the absorption spectra were recorded. DLS was used to determine the particle size, polydispersity index (PDI) and zeta potential analysis of the suspension placed in the appropriate cuvette.

Table 1
Ratio of reagent and time of reaction for the procedure of green synthesis by the high temperature method.

Zn $(NO_3)_2{\cdot}6H_2O$ (mg)	Extract (4.15 mg/ml) (mL)	T° reaction (°C)	Stirring time (Minutes)	Heating T° °C
1000	30	70	60	400
1000	30	70	15	400
100	30	70	15	400
100	35	70	15	400

Table 2
Conditions used for the low temperature method.

Zn $(NO_3)_2.6H_2O$ (0.1 M) (mL)	Extract (8.3 mg/ml) (mL)	T° reaction (°C)	Stirring time (Minutes)	Tween 80 (mL)	Gum arabic (g)	pH
22.5	5	70	60	1 –	– 0.28	10
22.5	5	70	60	1 –	– 0.28	9
22.5	5	70	60	1 –	– 0.28	11

The total phenolic content (TPC) expressed in pyrogallol was chosen as a tracer for phytocompounds (i) contained in the aqueous extract used for the green synthesis and (ii) capped on the synthesized ZnONPs obtained from each method. The TPC was determined according to the procedures described in the European Pharmacopoeia 10.8. In brief, 15 mL of water was added to 45 mg of the plant extract and each of the synthesized ZnONPs in separate 25-mL flasks. The mixtures were heated in a water bath for 30 min and cooled under running water. Then, they were transferred to another 25-mL flask, diluted to 25 mL with water and filtered with filter paper that was 125 mm in diameter. The first 5 mL of the filtrates were discarded, and 5 mL of each filtrate was diluted to 25 mL with water. Two milliliters of each of these solutions were mixed with 1 mL of phosphomolybdotungstic reagent and 10 mL of water and diluted to 25 mL with a 290 g/L solution of sodium carbonate decahydrate, and then they were kept out of the light for 30 min. As a standard, 25 mg of pyrogallol was dissolved in water and diluted to 50 mL with the same solvent. Five milliliters of this solution was diluted to 100 mL with water. Then, 2 mL of this solution was mixed with 1 mL of phosphomolybdotungstic reagent and 10 mL of water, diluted to 25 mL with a 290 g/L solution of sodium carbonate decahydrate and kept for 30 min in the dark. Next, 200 µL of each of these solutions (sample test and standards) were pipetted on a 96-well plate, and the absorbances were measured at 760 nm (A_1 for each sample test and A_2 for the standard) using water as the compensation liquid on the Spectramax® ID5 (Molecular Devices, LLC, USA). The percentage content of phenolic compounds in each sample was calculated from the equation (2):

$$\text{TPC} = \frac{\text{Df.A1.m2}}{\text{A2.m1}} \text{x100} \quad (2)$$

where m_1 = mass of the sample examined, m_2 = mass of pyrogallol in its last dilution, and Df = dilution factor (=62.5 in our case).

The different samples were compared using the results of the analysis.

2.7. Stability studies

The stability of the nanoparticles was investigated in biomimetic gastrointestinal fluids (fasted state simulated gastric fluid (FaSSGF), fed state simulated gastric fluid (FeSSGF), fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF)) prepared according to the methods and composition previously described (Duan et al., 2021; Xu et al., 2020), with some adaptations. In brief, 50 mg of ZnONPs prepared by low-temperature methods were dispersed in 5 mL

of water contained in different vials. Then, 0.1 mL of each of the resulting suspensions was diluted to 5 mL with water or each of the biomimetic gastrointestinal media and maintained under magnetic stirring (200 rpm, 37 °C) to mimic realistic gastrointestinal conditions. The size of the nanoparticles was then measured at different time points within 2 h (0, 0.5, 1 and 2 h) for the nanoparticles dispersed in simulated gastric fluids and within 6 h (0, 0.5, 1, 2, 3 and 6 h) for those dispersed in simulated intestinal fluids.

2.8. Yield of the green synthesis

The yield of ZnONP synthesis was calculated after complexometric titration of zinc ions according to the European Pharmacopoeia 10.8. Briefly, 0.150 g of ZnONPs was dissolved in 10 mL of dilute acetic acid (composed of 12 g of glacial acetic acid diluted to 100 mL with water), introduced into a 500-mL conical flask and diluted to 200 mL with water. Then, 50 mg of xylenol orange triturate (constituted by 1 part of xylenol orange and 99 parts of potassium nitrate) and hexamethylenetetramine were added until the solution became violet-pink. Then, 2 g of hexamethylenetetramine in excess was also added. The mixture was titrated with 0.1 M EDTA disodium until the violet-pink color shifted to yellow. The amount of zinc or zinc oxide was calculated according to the following relation: 1 mL of 0.1 M sodium edetate is equivalent to 6.54 mg of Zn or to 8.14 mg of ZnO. The yield of Zn was calculated by the following equation (3):

$$\text{Yield}\,(\%) = \frac{quantity\ \ of\ \ zinc\ \ in\ \ the\ \ \text{Zn}ONPSX100}{Quantity\ \ of\ \ zinc\ \ in\ \ the\ \ \text{Zn(NO3)2 .6H2O}used} \quad (3)$$

2.9. Oral glucose tolerance tests

All animal experiments were approved by and performed in accordance with the local animal committee (2022/UCL/MD/035) and as recommended by the Belgian Law of 29 May 2013 on the protection of laboratory animals. Two oral glucose tolerance tests (OGTTs) were carried out in 25.70–34.55 g normoglycemic and 25.67–44.05 g high-fat diet (HFD)-fed mice (C57BL/6J male mice, 13 weeks; Janvier Laboratories, France). HFD mice were fed a 60 % fat and 20 % carbohydrate (kcal per 100 g) diet (D12492i, Research Diets, USA) to evaluate the hypoglycemic capacities of the extracts of the most antioxidant plant and the ZnONP formulations that of different particle sizes. Before the experiments, mice were housed four per cage under day/night cycle of 12 h/12 h, temperature 20–24 °C, relative humidity 55 ± 5 % and underwent 2 weeks of acclimation under a normal diet, followed by 3 weeks of HFD (or normal diet in the case of healthy control mice). They had a free access to food and water.

2.9.1. OGTT in the mice treated with the plant extracts

The mice were divided into five different groups (8 mice/group): control normal diet (CTL-N), control HFD mice (CTL-HFD), mice subjected to aqueous extract administration (Mice Aq), mice subjected to DCM extract administration (Mice DCM) and mice subjected to DCM extract from the aqueous extract (Mice DCM-Aq). All mice were fasted 4 h prior to the OGTT and weighed, and 30 min before the OGTT, blood was collected from the tip of the tail vein using a heparinized capillar (60 μL) for insulin analysis (Ultrasensitive Mouse Insulin ELISA, Mercodia, Sweden) and glycemia checked (Accu-check Aviva glucometer Roche, Switzerland). Then, 15 min before the OGTT, CTL-N mice received 5.7 μL/g body weight of the vehicle (water, Tween 80, ethanol 90:7:3); CTL-HFD mice received 5.7 μL/g body weight of the vehicle; Mice Aq, Mice DCM & Mice DCM-Aq received 5.7 μL/g body weight of the extracts corresponding to a 400 mg/kg dose of crude extract. At time 0, glycemia was checked, and all mice received a glucose solution (2 g/kg) by gavage. Then, 15 min after receiving glucose, blood was collected from the tip of the tail vein as before for insulin analysis and glycemic control. The glycemia was checked again at 30, 60, 90 and 120 min after glucose gavage. The variation in glycemia was calculated in % with respect to fasted values.

2.9.2. OGTT in mice treated with ZnONPs

This test was conducted as per the extracts, except that green synthesized ZnONPs that involved the use of Tween 80 were administered instead. The ZnONPs administered presented a size of 560 nm, zeta potential −24.1 mv, PDI 0.24; or 480 nm, zeta potential –22.6 mv, PDI 0.19 and 240 nm, zeta potential –23.3 mv, PDI 0.14, at the equivalent dose of 10 mg ZnO/kg. The different ZnONPs groups, presenting different sizes, were named ZnONP-560, ZnONP-480 and ZnONP-240.

2.10. Statistical analysis

GraphPad Prism 9.1.2 software (San Diego, CA, USA) was used to run the statistical analysis. The Shapiro − Wilk test was used to check the normality of the samples. ANOVA followed by Tukey's post hoc test was applied to investigate the differences among groups. The *t* test was used to compare two groups. *P* values lower than 0.05 were used as the criteria for statistical significance. All tests were conducted in triplicate and are expressed as the mean ± standard error of the mean (SEM), unless otherwise stated.

3. Results and discussion

3.1. DPPH scavenging effect (% reduction)

Eighteen extracts were obtained from the different plants (three extracts from each plant sample). The percentage of DPPH reduction by each of these extracts was analyzed to select the best plant for ZnONP synthesis (which occurs through the reduction of zinc ions by the electron-donating functional groups of plant compounds). The percentage of DPPH reduction, i.e., the antioxidant activity of the extracts, indicated their capacity to biosynthesize ZnONPs capped with antioxidant phytochemicals. Since hyperglycemia progressively induces oxidative stress, which is involved in several complications, these antioxidant phytochemicals reduce oxidative stress and are known to mitigate the complications of diabetes (Kambale et al., 2022; Rahimi et al., 2005). All the extracts showed antioxidant activity by decolorization of DPPH from purple to yellow. As shown in Fig. 1, *P. oleosa* extracts (aqueous, ethanol 9 %: water at 50:50 and ethanol 99 %) showed significantly higher DPPH scavenging effects (higher reducing power) than the other plant extracts. Hence, *P. oleosa* was chosen for the synthesis of ZnONPs, given that the probability of synthesizing more efficient ZnONPS was higher by using the plant with higher antioxidant and/or high reducing potential. The aqueous medium is the most commonly used for green synthesis, and *P. oleosa* aqueous extracts were used for this purpose in this study.

3.2. Characterization of ZnONPs

ZnONPs were synthesized by mixing zinc nitrate hexahydrate with *P. oleosa* aqueous extract while controlling the parameters described in Tables 1 and 2. Cloudiness was instantly observed in the medium, indicating the beginning of ZnONPS synthesis. The successful synthesis was confirmed by different analyses. The UV–visible absorption wavelength of ZnO in the (nano)particulate form of the aqueous dispersions of all the synthesized ZnONPs was examined by means of a spectrophotometer by scanning from 200 to 800 nm (Supplementary information, Figure S1). For all the synthesized ZnO particles, the presence of their characteristic absorption peaks at 354 nm was observed, which was within the range of 300 to 400 nm and in accordance with the literature (Rajakumar et al., 2018; S. Naseem Shah et al., 2016). The ZnO particles resulting from the high-temperature method showed lower peak heights. In contrast, those obtained from the low-temperature method showed higher peak heights at pH 9 and 10

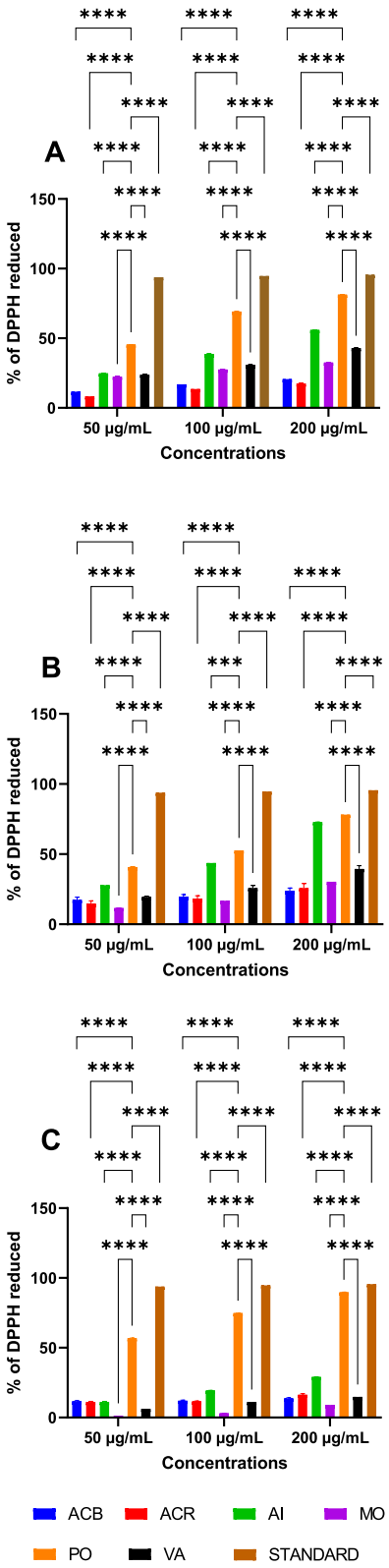

Fig. 1. DPPH scavenging effect (% reduction) of the different extracts obtained using (A) water, (B) ethanol 99 %:water at 50:50 and (C) ethanol 99 %. Data are presented as the mean ± SEM (n = 3). *P* values were determined by two-way ANOVA followed by a Tukey post-hoc test. Legend: ACB: (*Alium cepa*, white onions); ACR: (*Alium cepa*, red onions); AI: *Azadirachta indica*; MO: *Moringa oleifera*; PO: *Panda oleosa*; VA: *Vernonia amygdalina.* * PO extracts *vs.* other extracts in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to the peak observed at pH 11, which was very low. This indicated that the process using low temperature (pH 9 to 10) produced nanoparticles with more surface electrons that absorbed more UV light and suggested a large surface area, i.e., smaller nanoparticles than those obtained at high temperature. We could conclude that the synthesized (nano)particles showed a dependence on the method used for their synthesis.

The nanoparticle analysis by dynamic light scattering showed sizes of 423 ± 65.50 nm, PDI ≥ 0.32 ± 0.01 and a zeta potential of approximately −29.4 ± 0.21 mV for the ZnONPs obtained with the high-temperature method. The nanoparticle analysis from the low temperature method showed an average size of 201 ± 17.83 nm, PDI of 0.15 ± 0.03 and a zeta potential of –23.2 ± 0.65 mV. This indicated that Tween 80 or gum arabic contributed to the reduction in the size of the nanoparticles. In addition, this lower PDI indicated that the suspending agents contributed to more homogenous ZnONPs in size. The difference observed in zeta potentials between ZnONPs obtained by the high-temperature method and the low-temperature method may be due to the capped phytochemicals on the nanoparticles from the latter.

3.3. Phytocompound content in the plant extracts and ZnONPs

Given that the plant extract can contain several metabolites, including phenolic compounds, these were analyzed as tracers of phytochemicals due to their reducing activity. According to the procedures described in the European Pharmacopoeia 10.8, the test sample used from the plant extract and each of the synthesized ZnONPs was 45 mg (m1) and was diluted 62.5 times (dilution factor (Df)). The mass of the standard (pyrogallol) (m2) in its last dilution was found to be 0.05 mg. The results of the total phenolic compound (TPC) expressed in the pyrogallol analysis are summarized in Table 3 below, showing that the aqueous extract of *P. oleosa* had a TPC of 11.58 ± 0.21 %. These phenolic compounds were almost unrecovered in the ZnONPs obtained via the high-temperature method (0.75 ± 0.06 %). However, they represented 2.58 ± 0.04 % and 2.56 ± 0.03 of the ZnONPs from the low-temperature method using Tween 80 and gum arabic, respectively. The ZnONPs from the high-temperature process lost more than 99 % of the phenolic compounds, while those from the low-temperature method retained 92.90 % and 92.10 % of the phenolic compounds used for their synthesis (Supplementary information, Table S1). These results indicated that high temperature destroys phytocompounds, but freeze-drying preserves them. The low loss of phenolic phytocompounds by the freeze-drying method can be attributed to the washing of the nanoparticles before freeze-drying. Since there was a significant loss of phytocompounds with the high-temperature method, the low-temperature method was preferred, as the retained phytocompounds would contribute to improving the activity of ZnONPs.

3.4. Stability of ZnONPs

A stability study of the synthesized ZnONPs was carried out to predict their behavior in the gastrointestinal tract after oral administration. Simulated gastrointestinal media were prepared for this purpose. To assess the stability in these biomimetic media, the synthesized ZnONPs following the low temperature method were dispersed in water prior to

Table 3
Total phenolic content of the aqueous extract and ZnONPs (n = 3, mean ± SEM).

	P. oleosa aqueous extr	ZnONPs from high temperature method	ZnONPs from low temperature methods (using Tween 80 or gum arabic)
TPC (%)	11.58 ± 0.21	0.75 ± 0.06	2.58 ± 0.04 and 2.56 ± 0.03
Phytocompound retention (%)	–	0.01	92.90 and 92.10

dilution in gastrointestinal simulated media. During the course of the study, the nanocarriers kept their size intact when dispersed in water regardless of the use of Tween 80 or gum arabic within their preparation. However, we observed that the ZnONPs synthesized with Tween 80 exhibited a decrease in size, remaining below 185 nm in FaSSGF, FeSSGF and FeSSIF and exhibiting a remarkable increase from time 0 in FaSSIF. The nanoparticle sizes in this medium remained elevated with fluctuations over time (Fig. 2A). When compared with the ZnONPs prepared with gum arabic, these showed a considerable decrease in size only in FeSSIF and an increase with fluctuations in the other simulated media from time 0 (Fig. 2B). This showed that in the FaSSIF, all the ZnONPs were unstable. On the one hand, this increase in size might result from an aggregation of the ZnONPs, which can lead to a low absorption of zinc, and on the other hand, the decrease in size can lead to an increase in their absorption in the gastrointestinal tract. The ZnONPs prepared with Tween 80 were chosen for the OGTT because they exhibited less or no tendency to aggregate in these biomimetic media.

3.5. Quantification of zinc oxide

As the green-synthesized ZnONPs are made of ZnO and capping agents, the analysis of the proportion of ZnO makes it possible to determine the yield from its synthesis and to administer a precise and known quantity of zinc or ZnO. The complexometric titration of zinc was realized as described in the European Pharmacopoeia 10.8 from the synthesized ZnONPs involving Tween 80. This study was conducted in triplicate. The results showed that in 150 mg of test sample, zinc oxide accounted for 125.89 ± 1.08 mg, which represented 84 ± 0.72 % of the analyzed sample. This confirmed that the synthesized ZnONPs were formed by zinc oxide capped by phytochemicals, which included phenolic compounds. Referring to the European Pharmacopoeia 10.8, 0.1 M zinc nitrate hexahydrate corresponds to 6.538 g of zinc/L or 8.138 g of ZnO/L. Given that 22.5 mL of a 0.1 M solution was used for the green synthesis, they were expected to yield 147.1 mg of zinc or 183.1 mg of ZnO. The resulting ZnONP weight was 173.86 ± 4.9 mg. Taking into account that the complexometric titration of zinc showed that zinc oxide accounted for 84 ± 0.72 % of the green-synthesized ZnONPs, the amount of zinc oxide corresponded to 146.04 ± 2.96 mg.

The yield of zinc or zinc oxide when compared to its amount in zinc nitrate hexahydrate that was involved in the synthesis was therefore approximately 79.76 ± 1.07 %. This result shows that most of the zinc was not lost during the ZnONP synthesis.

3.6. Hypoglycemic activity

The hypoglycemic activities of *P. oleosa* extracts and green-synthesized ZnONPs were assessed in mice to compare their therapeutic effects against hyperglycemia. *Panda Oleosa* extracts have not yet previously investigated against hyperglyemia in mice before. There is a lack of data available on their effect in this model. In the current study, the analysis of fasting glycemia and fasting insulinemia (Fajardo et al., 2014) confirmed that the HFD-fed mice had developed hyperglycemia before glucose administration but were not yet associated with insulin resistance (Parks et al., 2015) as shown in Supplementary Figure S2 and S3. During the OGTT studies, we first analyzed the antihyperglycemic activity of different extracts (aqueous extract, DCM extract from the aqueous extract and DCM extract) of *P. oleosa* alone. An oral administration dose of 400 mg/kg was selected for each *P. oleosa* extract, as this dose had already been described in the literature for the extracts of other plants cited in this work (Atangwho et al., 2014; Attakpa et al., 2017; Faisal et al., 2014; Patil et al., 2013). As a result, only the dichloromethane extract was able to significantly reduce the area under the curve (AUC) when compared to the CTL HFD group (Fig. 3A&B). The aqueous extract did not show a significant reduction in mice in contrast to what was obtained in rabbits by Muhoya et al., in an OGTT study (Muhoya et al., 2017). This could be due to difference between the two animal models.

Concerning the synthesized ZnONPs, a dose corresponding to 10 mg ZnO per kg, as previously reported in the literature (El-Gharbawy et al., 2016; Siddiqui et al., 2020), was administered once. This dose avoided a strong increase in glycemia in treated mice after glucose administration, as shown in Fig. 4A. The AUC in Fig. 4B shows that ZnONP-240 exhibited significant antihyperglycemic activity. This result is in agreement with Bala et al. who showed that green-synthesized zinc oxide nanoparticles presenting a smaller particle size showed a more significant antidiabetic activity than those of larger size (Bala et al., 2014).

After glucose administration, an increase in blood glucose was observed in all the mice followed by a tendency toward restoration after approximately 2 h. This increase in blood glucose levels was not significantly different from that of normal mice in the DCM extract of PO- and ZnONP-treated mice and was significantly lower compared to that of HFD controls (Fig. 3A and 4A). Pretreatment with ZnONPs (corresponding to 10 mg of ZnONPs capped with 0.31 mg of phenolic compounds (Supplementary information, Table S1)) preserved the strong rise in blood glucose (Fig. 4), and the effect was comparable to the effect exerted by a high dose (400 mg/kg) of the plant extract (Fig. 3). AUC was found to be significantly lower for the groups of mice receiving the *P. oleosa* DCM extract and the ZnONPs (Fig. 3B and 4B).

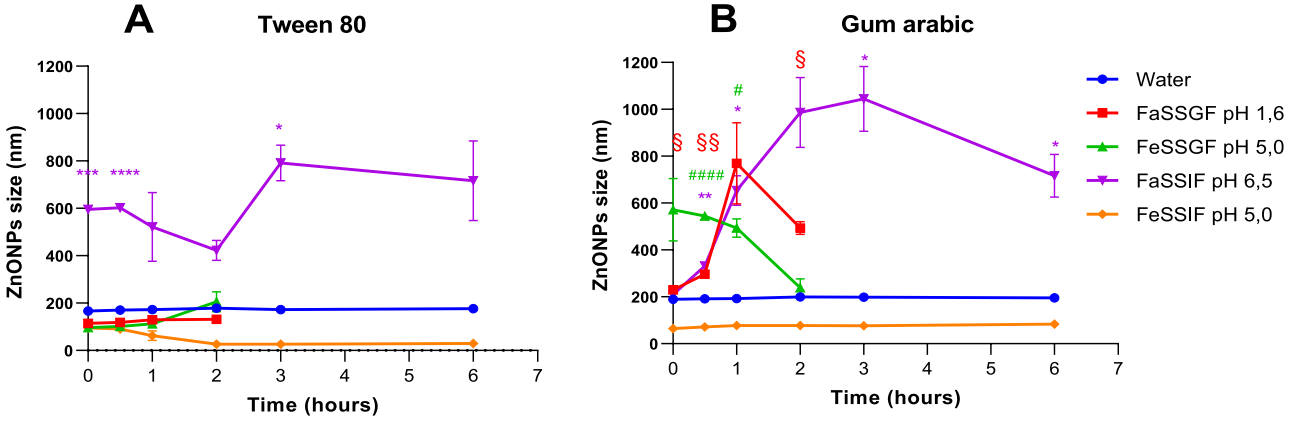

Fig. 2. (A) *In vitro* stability of PO-ZnONP prepared with Tween 80 in simulated intestinal fluids. (B) *In vitro* stability of PO-ZnONP prepared with gum arabic in simulated intestinal fluids. Data are presented as the mean ± SEM (n = 3). *P* values were determined by 2-way ANOVA. *FaSSIF *vs.* water; #FeSSGF *vs.* water; §FaSSGF *vs.* water.

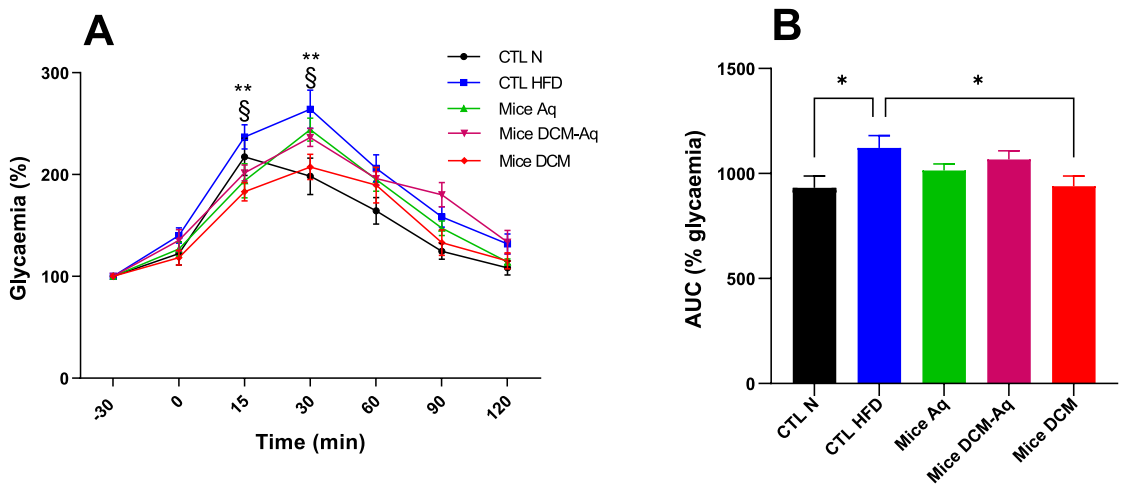

Fig. 3. Hypoglycemic activity of *P. oleosa* (PO) extracts evaluated via OGTT expressed as % with respect to the time of 30 min (A) and the corresponding AUC (B). P values were calculated by 2-way ANOVA (A) or one-way ANOVA (B) (mean ± SEM; n = 8). §CTL HFD *vs.* Aq extr of PO; *CTL HFD *vs.* DCM extr PO.

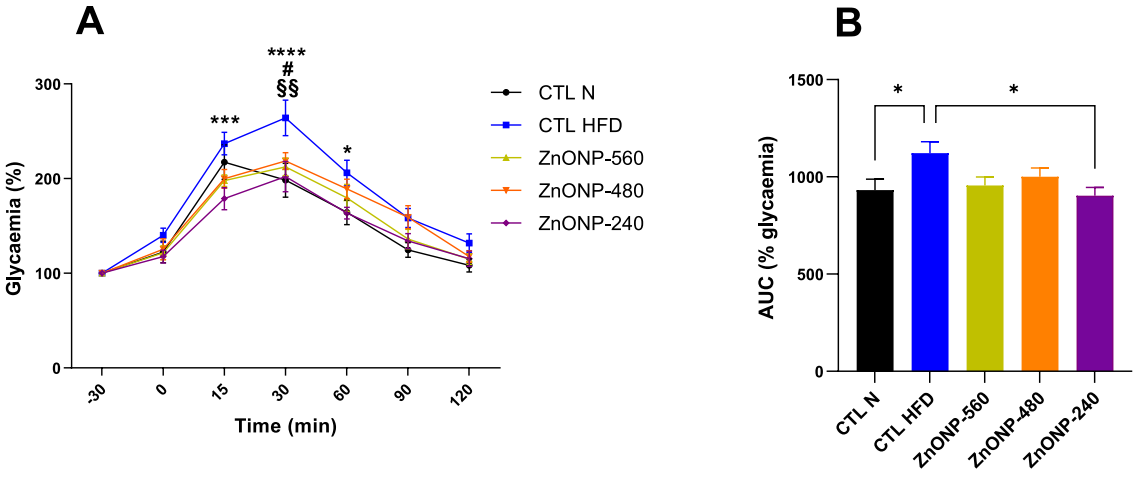

Fig. 4. Hypoglycemic activity of ZnONPs evaluated via OGTT expressed as % with respect to the time of 30 min (A) and the corresponding AUC (B). P values were calculated by 2-way ANOVA (A) or one-way ANOVA (B) (mean ± SEM; n = 8). *CTL HFD *vs.* ZnONPs-3; §CTL HFD *vs.* ZnONPs-1; #CTL HFD *vs.* ZnONPs-2.

4. Conclusion

The aqueous extract of *P. oleosa* was selected for the green synthesis of ZnO nanoparticles because of its strong antioxidant activity compared to the extracts of other plants evaluated. To our knowledge, this plant extract has not been evaluated before for the preparation of ZnONPs. A comparison of two green approaches for the preparation of ZnONPs (heating at high temperatures *versus* low temperatures with the addition of suspending agents including Tween 80 or gum arabic and with pH variations followed by freeze-drying) was carried out. The high-temperature synthesis approach led to nanoparticles that were almost uncapped by phytochemicals. The low-temperature method with the addition of suspending agents with adjustment of the pH of the reaction medium between 9 and 10 allowed us to obtain nanoparticles with high retention of phytochemicals. These nanoparticles were more stable when Tween 80 was used in their manufacture than when gum arabic was used. Pronounced antihyperglycemic activity was observed. From these results, we demonstrated that ZnONPs with a high retention rate of phytochemicals (phenolics) are able to induce antihyperglycemic effects at a lower dose compared to the extracts alone (10 mg ZnO/kg + 0.31 mg phenolic compounds/kg *vs.* 400 mg plant extract/kg (where only the DCM extract was effective, not the aqueous one)). This strategy could represent an easier, greener and more affordable strategy for the management of, e.g., type 2 diabetes mellitus.

CRediT authorship contribution statement

Espoir K. Kambale: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft. **Frederick M. Katemo:** Investigation, Resources. **Joëlle Quetin-Leclercq:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. **Patrick B. Memvanga:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. **Ana Beloqui:** Conceptualization, Data curation, Methodology, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122715.

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