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Improvement of Phenolic Compounds and Antihyperlipidemic Activity of *Hibiscus sabdariffa* L. Calyxes Powder Using CDS Processing

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Abstract: The aim of this study was to evaluate the effect of controlled differential sieving processing (CDSp) on the content of phenolic compounds and antihyperlipidemic activity of *H. sabdariffa* calyxes powder. For this, four granulometric classes (<180 µm, 180-212 µm, 212-315 µm, ≥315 µm) were analyzed. Unsieved powder and lyophilized ethanolic extract were used for comparison. First, we identified and quantified the phenolic compounds present in the samples by chromatography in liquid phase coupled to a UV detector and mass spectrometry (LC-MS). Second, we evaluated the antihyperlipidemic activity of the different fractions in adult male rats at a dose of 250 mg/kg and compared them with those of unsieved powder and the ethanolic extract. LC-MS analysis identified seven compounds (quercetin, rutin, catechin, ferrulic acid, p-coumaric acid, protocatechic acid and cafeic acid) in the different sample of *H. sabdariffa* calyxes powder. The granulometric class 212-315 µm showed the best concentration, higher than those of the other granulometric class and crude powder. This fraction also showed content of phenolic compounds higher than those of ethanolic extract except for p-coumaric acid, and protocatechic acid which were concentrated equally in both samples. Antihyperlipidemic activity were significantly influenced ($P < 0.05$) by particle sizes. The best activity was obtained in the particle size classes of 212-315 µm and <180 µm which were significantly ($P < 0.05$) higher than those of the other fractions and of the unsieved powder to improve the lipid parameters (total cholesterol, LDL-C, HDL-C and triglycerides), transaminases (ALAT and ASAT) and creatine. However, compared to the ethanolic extract, only the granulometric class of 212-315 µm showed a significantly higher activity. CDSp can be an alternative to ethanol extraction and may be an option to improve the content of phenolic compounds and antihyperlipidemic activity of plant powders.

Keywords: Antihyperlipidemic Activity, *Hibiscus sabdariffa* Calyxes, Powder Fraction, Lyophilized Ethanolic Extract, Phenolic Compounds

1. Introduction

Risk factors for cardiovascular disease (CVD) are parameters for which an individual's exposure to them increases the risk of disease occurrence, while their elimination or improvement decreases this risk [1]. Among these risk factors, we have hyperlipidaemia, which refers to a pathological condition characterised by elevated LDL-cholesterol (LDL-C), total cholesterol (TC), and/or triglycerides (TG) as well as decreased HDL-cholesterol

(HDL-C) [2]. It is responsible for 56% of CVD cases, the frequency of which is increasing worldwide [3]. The current management of hyperlipidaemia is based initially on simple dietary hygiene measures in the individual. In a second phase, if these prove insufficient, drug treatments will be prescribed in combination with the continuation of the diet [4]. There are various lipid-lowering treatments. The main ones are HMG-CoA reductase inhibitors or statins, fibrates, bile salt chelating resins. However, most patients subjected to the action of these drugs face toxic effects characterised by progressive liver disease, severe renal failure, gout, hyperglycemia, myolysis, diarrhoea and gastric irritation [5]. In view of these observations, the use of plants therefore appears to be an alternative to synthetic products. Indeed, several studies have shown that plants have antihyperlipidemic properties [6, 7]. It is in this craze that we chose Hibiscus sabdariffa L. which is a herbaceous plant of the Malvaceae family widely distributed in the tropics and subtropics [8]. It is used in several parts of Africa for food and medicinal purposes for its calyxes, leaves and seeds. However, the calyxes are the most widely used part of the plant. It is also used in the preparation of the local drink known as "folére" in the northern regions of Cameroon. Several studies have reported that this plant contains numerous bioactive molecules that have many medical and biological effects [9, 10]. To be valorized, these active compounds are usually extracted from the original plant matrix. Thus, the extraction of active principles from the plant material, such as phenolic compounds, which are currently attracting increasing interest due to their role as natural antioxidants, is a crucial step: it determines the quantity and nature of the extracted molecules, on which the expected biological properties depend [11].

Plant extract is commonly obtained by standard solvent methods and generally, ethanol is the most commonly used solvent due to its high extractability of phenolic compounds [12]. Despite its efficiency, this technique has limitations that include the use of a large amount of energy, the release of large amounts of residues and the use of solvents that have adverse consequences affecting, among others, health, the environment as well as the active [13]. These limitations have led to criticism from consumers and industries and are paving the way for the development of new alternative methods with significant advantages over solvent extraction. This has led to the development of the Controlled Differential Spray process (CDSp), which reduces these collateral effects, while ensuring a safe and high quality natural extract. CDSp is a new dry extraction process that differs from other conventional processes in that it uses no organic solvents, is harmless to humans and the environment and is readily available for the production of a wide range of active ingredients of very different sizes and molecular weights [14]. The efficiency of this extraction process has been demonstrated by several studies [15-17]. However, the efficiency of this process compared to solvent extraction methods has not been clarified. The present work therefore aims to evaluate the effect of powder particle size on the phenolic content and antihyperlipidemic activity of *H. sabdariffa* compared to ethanol extraction.

2. Materials and Methods

2.1. Reagents and Plant Materiel

HPLC grade acetonitrile, water, methanol, ethanol Protocatechic acid, caffeic acid, epicatechin, ferrulic acid, rutin, quercetin, p-coumaric acid, catechin, chlorogenic acid and formic acid were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), transaminases (ALAT and ASAT) and creatinine measuring kits were obtained from (ENSAI) and used according to manufacturer's instructions procedures. The plant material used in the present study consists of dried calyxes of *H. sabdariffa*. This plant material was purchased from the local market in the Adamaoua region of Cameroon. They were cleaned by hand to remove foreign bodies (inorganic materials, dirt and dust particles) before being ground for powder production.

2.2. Grinding of Dried Calyxes and Sieving of Powder

The CDSp procedures performed to obtain the powder fractions were similar to those reported in the previous studies by Deli *et al.* [10]. He is based on the separation of particles from a granular material by making them pass through several sieves of decreasing mesh size (315, 212, and 180µm in the current study). 100g ground plant sample is sieved in permanent vibratory mode at 0.5 mm amplitude for 10min. The fraction of the powder retained on each sieve is recovered and weighed for the calculation of the mass fraction of each granulometric fraction. In this study, an electric Ultra-Centrifugal Mill ZM 200 (Haan, Germany) supplied with 24-tooth rotor of 99 mm and trapezoid holes a mesh sieve of 1 mm and operated at 12,000 rpm was used to make whole powder of dried calyxes. Then ground material was sieved with the Analysette 3 Spartan apparatus (Fritsch, Idar-Oberstein, Germany), operating by vertical vibration. The following powder fractions were obtained: <180µm, 180–212 µm, 212–315 µm, and ≥315 µm, with moisture content of about 10–12%. Unsieved powder was taken as control, and the powdered samples were stored at 10°C in polyethylene bags and placed at room temperature (25 ± 2°C) until they were used.

2.3. Production of Ethanolic Extract Powder

A mass of *Hibiscus* powder was weighed using a scale (Gibertini brand) and macerated with ethanol 95° in the ratio 1:10 (m/v) with stirring (electric stirrer) for 24h in order to homogenize the mixture. The homogenate obtained was then filtrated with Whatman No 1 filter paper (pore size 12–15 lm) and ethanol was removed using a rotary evaporator (BUCHI - R210/215) at 40°C and under reduced pressure of 17.500Pa. The frozen extract (- 18°C) was put into the freeze dryer chamber for 48 h and under pressure of 10Pa. The temperature of freeze dryer was at – 60°C. Collected powder

extract was conditioned in polyethylene bags and stored at 10°C until analysis.

2.4. LC-MS Analyses

2.4.1. Extraction of Phenolic Compounds

For the preparation of the extracts, 2g of each powdery fraction was mixed in 20ml of water-methanol mixture 30:70 (v/v) by stirring (300 rpm) at room temperature of the laboratory (18 ± 2°C) for 24 hours. This maceration technique makes it possible to extract all the phenolic compounds present in the matrix and achieve the same extraction yield for small and large particles [17]. It does not involve heat treatment and no acid addition likely to cause degradation of the compounds. Subsequently, the hydro-alcoholic extracts were filtered through a filter paper. The supernatant was recovered and the total volume was adjusted to 15mL with the extraction solvent and stored in a cold room at 4°C for analysis.

2.4.2. LC-MS Analytical Conditions

LC-MS analyses were performed on a LC-MS 2020 system (Shimadzu, Tokyo, Japan) associated with Electrospray Source Ionization (ESI). The separation was carried out on 150 × 4.6 mm. C18 inverse phase Gemini column (Phenomenex, Torrance, CA, USA) with a particle size of 3 µm and a pore size of 130Å. The column mixture was set at 35°C. The mobile phase composed of (A) 0.5% acetonitrile in water and (B). The injection volume was 20 µl and the flow rate was 0.6 ml / min. The following elution gradient was used: 0-10 min, 10-15; 10-15 min, 15-20; 15-18 min 20-25; 18-22 min, 25-60; 22-25 min of 60:60; 25-28 min, 60-10; 28-30, control (equilibration step). This gradient further comprises a rinsing phase and a rebalancing phase of the column. The electrospray ionization source was used in negative mode. The nebulization gas flow rate was set at 1.5L/min, the gas flow rate at 20L/min, the thermal block temperature was stopped at 350°C, and the temperature of the desolvation line (DL) at 250°C. Double detection was ensured by a photodiode array detector (PDA) in the 200 – 400 nm wavelength range and mass spectrometry (MS). MS settings were as follows: negative mode electrospray source (ESI), 1.5L/min nebulization gas flow, 15 L/min drying gas flow, 300°C heat block temperature, 250°C desolvation line temperature, -4 kV probe voltage, and selected-ion monitoring (SIM) acquisition.

2.4.3. Calibration Method

The identification of phenolic compounds in plant extracts was based on standard compound analyses confronting m/z ratio and retention time (tr). For quantitative analysis, a five-level linear calibration curve was obtained by injection of known concentrations (from 10^{-4} to 10^{-2} mg/mL) of standards. Method sensitivity was assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to signal-to-noise (S/N) values of 3 and 10, respectively. The following calibration parameters were obtained: Chlorogenic acid (R^2 = 0.997; LOD= 2.2 ppm; LOQ= 7.0 ppm); Epicatechin (R^2 = 0.999; LOD= 1.9 ppm; LOQ= 8.3 ppm); catechin (R^2 = 0.998; LOD= 2.3 ppm; LOQ= 8.0 ppm); Quercetin (R^2 = 0.991; LOD= 1.9 ppm; LOQ=6.3 ppm); cafeic acid (R^2 = 0.994; LOD= 2.1 ppm; LOQ= 6.8 ppm); P-coumaric acid (R^2 = 0.997; LOD= 2.3 ppm; LOQ= 7.7 ppm); Ferrulic acid (R^2 = 0.995; LOD= 2.2 ppm; LOQ= 7.4); Protocatechic acid (R^2 = 0.997; LOD= 1.3 ppm; LOQ= 3.7 ppm) and Rutin (R^2 = 0.998; LOD= 0.3 ppm; LOQ= 0.9 ppm). Results were expressed in mg of standard per gram of dry matter.

2.5. Animals and Induction of Hyperlipidemia

The complete experiment was carried out using 45 wistar male rats of albino species (*Rattus norvegicus*) of weighing between 250-275g. These animals were assimilated to the animal factory of the National School of Agro-Industrial Sciences (ENSAI) of the University of Ngaoundere. They were housed 5 per cage in standard environmental conditions (25±5°C) and fed with food and water. All the animals were exposed to an alternate cycle of 12 h of darkness and 12 h of light. The mice were acclimatized to the laboratory conditions for 10 days prior to initiation of the experiment.

The induction of hyperlipidemia was done in animals by the administration of a fat diet. It was made according to the method describe by Hamlat *et al*. [18]. The normal diet is composed of 20% protein, 64% carbohydrate and 5% fat. While the high-fat diet is composed of 10% protein, 25.3% carbohydrate and 60% fat. Studies have shown that diets rich in lipids can cause the onset of hyperlipidemia.

2.6. Experimental Procedure

During 28 days of experimentation, the animals were fed and treated simultaneously based on the different fractions and ethanolic extract of *H. sabdariffa*. The samples were macerated in distilled water and stirred continuously for 2 hours to homogenize the mixture. Every morning at the same time a volume of 10 mL / kg was administered by gavage using an endogastric tube at a dose of 250 mg per kg of body weight. The rats were randomly divided into 9 groups of 5 rats: normal control group, negative control group, positive control group, and 6 experimental groups who receiving powder fractions of <180µm, 180-212µm, 212-315µm, and ≥315µm, unsieved powder and ethanolic extract powder. The normal control group was submitted to normal food regime while the others received high-fat diet. Before foods being given to rats, they were administered per os 1 h before 10 mL/kg of aqueous suspension of *H. sabdariffa* powder for the experimental groups, Atorvastatin for the positive control group, and distilled water for the negative controlled group. *H. sabdariffa* powders and ethanolic extract were administered at the dose of 250mg/kg body weight, while atorvastatin was administered at the dose of 10mg/kg body weight.

2.7. Measurement of Food Intake, Weight Gain and Faecal Lipid

The food is left at their disposal all day and the remains are

collected and quantified to deduce the exact amount of food consumed by the different groups. The rats were weighed from the beginning of the experiment, then every 2 days until the end of the four weeks of study. The initial and final weights of the rats were reported as well as the daily food intake of each group. These data enabled us to calculate the weight gain (ΔP). The lipid content of feces was evaluated after extraction with Soxhlet by hexane according to the Russian method described by [19].

$$\Delta P = P_f\text{-}P_i$$

With
ΔP: $gain\ (g)$
P_i: $initial\ weight\ (g)$
P_f: $final\ weight\ (g)$.

2.8. Sacrifice and Blood Collection

At the end of the experiment, all the animals were fasted for 12 hours. They were then anesthetized by inhalation of diethyl ether and blood was immediately drawn into heparin tubes after incision of the neck. The blood was collected in heparin tubes, allowed to stand for 4 hours at room temperature and then centrifuged at 3000rpm for 10 min at 25°C. The serum was collected and then stored in dry tubes and kept at -20°C for the analysis of biochemical parameters. The liver, kidneys, brain, testicles and heart were removed. For each rat sample weighed, the organs liver, kidneys, brain, testicles and heart were also weighed and the ratio of organ to body weight allowed us to obtain the organ index (OI) according to the following formula.

$$\text{OI} = (\frac{M_{Org}}{M_{cor}}) * 100$$

with
OI: $organ\ index$
M_{Org}: $organ\ mass\ (g)$
M_{cor}: $body\ mass\ (g)$

2.9. Determination of Lipid Profile Parameters

2.9.1. Total Cholesterol (TC)

CT was determined according to the enzymatic method described by Naito *et al*. [20]. The principle of this method is that under the action of cholesterol esterase, esterified cholesterol is converted into cholesterol and fatty acid. Oxidation of cholesterol in the presence of cholesterol oxidase produces cholesterol-3-one and hydrogen peroxide. Quinoneimine (pink) serves as an indicator which is formed by the action of hydrogen peroxide, 4-aminoantipyrine and phenol under the catalytic action of peroxidase. In the assay tubes, 10µL of serum and 10L of standard were introduced respectively. In these tubes, 1000µL of cholesterol reagent was added. After homogenization and incubation for 10 min at 25°C, the absorbance of the sample was read against the reagent blank at 505nm using a spectrophotometer. The TC concentration will be determined by the following formula:

$$\text{TC} = (\frac{D.O\ Sample}{D.O\ standard})*200$$

with
TC: $total\ cholesterol\ (mg/dL)$
D.O: $light\ density$.

2.9.2. HDL-Cholesterol (HDL-C)

HDL-C was determined by the enzymatic method described by Gordon *et al.* [21]. Chylomicrons and very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were precipitated by the addition of phosphotungstic acid and magnesium chloride. The supernatant obtained after centrifugation contains high density lipoproteins (HDL) which are determined using the total cholesterol reagent. 200µL of plasma and 500 µL of precipitation reagent were added to the centrifuge tubes. After homogenization, the tubes were allowed to stand for 10 minutes at room temperature, then centrifuged at 4000rpm for 10 minutes and the supernatant was collected. In the assay tubes, 100 µL of standard and 10L of serum were introduced. To these tubes 1000µL of cholesterol reagent was added. After homogenization and incubation of the tubes for 10 minutes, the optical density was read at 500 nm against the blank. The HDL-C concentration of the sample is calculated according to the following formula:

2.9.3. Triglycerides (TG)

TG were determined according to the method described by Fossati *et al*. [22] with some modifications. This method is based on the principle that under the action of lipoprotein lipases, glycerol produced by enzymatic hydrolysis of TG is phosphorylated by ATP to produce glycerol-3-phosphate and ADP through a reaction catalyzed by glycerolkinase. Glycerol-3-phosphate oxidase then catalyzes the oxidation of glycerol-3-phosphate to produce dihydroxyacetone-3-phosphate and H_2O_2. The latter combines with 4-aminoantipyrine and 4-chlorophenol to form quinoneimine under the catalytic influence of peroxidase. The intensity of the coloration is proportional to the concentration of TG present in the sample. In the assay tubes, 10µL of serum and 10L of standard were introduced respectively. In these tubes, 1000 µL of cholesterol reagent were added. After homogenization and incubation for 10min at 25°C, the absorbance of the sample was read against the reagent blank at 505nm using a spectrophotometer. The concentration of TGs in serum is given by the following relation:

$$\text{TG} = (\frac{D.O\ échantillon}{D.O\ standard})*250$$

with
TG: $Triglycerides\ (mg/dL)$
D.O: $optical\ density$.

2.9.4. LDL-Cholesterol (LDL-C)

LDL-cholesterol concentration was calculated from total cholesterol concentration, HDL-cholesterol concentration, and triglyceride concentration according to the following formula by Friedewald *et al*. [23]:

$$\text{LDL-C} = \text{TC} - \text{HDL-C} \left(\frac{\text{TG}}{5}\right)$$

with $LDL - C$: *cholestérol LDL* (mg/dL).

2.9.5. Atherogenicity Ratios

The TC/HDL and LDL/HDL ratios are the best indicators of the risk of cardiovascular diseases. The so-called normal values are less than or equal to 3.5 and 5 respectively for the LDL/HDL and TC/HDL ratios [24]. The ratios of LDL/HDL ratios or atherogenicity ratios are used to justify the predisposition of a subject to atherosclerosis.

2.9.6. Determination of Transaminases (ALAT and ASAT) and Creatinine

Creatinine

Creatinine was determined according to the method of Henry [25] and the Randox kit was used. The principle of this method is that creatinine in alkaline medium reacts with picrate to form a colored complex. In centrifugation tubes was introduced, 1 mL of trichloroacetic acid and 1 mL of plasma. The resulting solution was mixed, centrifuged at 2500rpm for 10 minutes and the supernatant was collected. In the white and standard tubes was introduced 0.5mL of trichloroacetic acid solution, then 1 mL of supernatant, 0.5mL, distilled water and 0.5 of standard solution were introduced respectively in the assay, blank and standard tubes in all these tubes was also added 1 mL of working solution which is V/V mixture of picric acid and sodium hydroxide. The mixture obtained in each tube is left to stand for 20 minutes at 25°C and the absorbance read against the blank at 520nm. The creatinine concentration of the sample (plasma) is calculated according to the following formula:

$$\text{C sample} = 2\text{x}\ \frac{\text{D.O.sample}}{2a\text{D.O.standard}}\ (\text{mg/dL})$$

ALAT Levels

The levels of alanine and aspartate aminotransferase were determined using the Randox kit according to the method of Reitman *et al*. [26]. The principle of this method is that ALAT transfers the amino group of alanine to α-oxoglutarate to form pyruvate and L-glutamate. With 2,4dinitrophenylhydrazine pyruvate forms pyruvate hydrazone. ALAT is determined by following the concentration of pyruvate hydrazone. In the tubes of assay and blank, are introduced respectively 0.1 mL of sample and 0.1 mL of distilled water, then we added in each of these tubes, 0.5 mL of buffer solution. The obtained solution is mixed and incubated at 37°C for 30 minutes. After incubation we also introduced into these tubes, 0.5 mL of 2,4-dinitrophenylhydrazine solution the mixture was made and the solutions incubated at 25°C for 20 minutes. In the obtained solutions, 5 mL of sodium hydroxide solution was introduced and the absorbance of the sample against the blank was read after 5 minutes at 546nm.

ASAT Levels

ASAT catalyzes the transfer of the amino group from L-aspartate to α-ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH under the action of MDH to give malate and NAD+. The concentration of NAD+ in the medium is proportional to the activity. ASAT substrate solution (0.1mL) was introduced into the blank and test tubes and preincubated at 37°C for 5 min, and then 0.02mL of serum was added to the test tubes. After incubation at 37°C for 1 h, 0.1mL of staining reagent was added. The tubes were left at room temperature for 20 min, and then the reaction was stopped by adding 1mL of NaOH. The optical densities were read at 505nm against the white tube.

2.10. Statistical Analyses

Each experiment and measurement was performed in triplicate. Results were reported as means ± standard deviations and statistical analyses were done using statistical package of Statgraphics 11.1. The following statistical tests were performed: one-way analysis of variance and multiple comparison of Duncan's test with significance defined at $p<0.05$. Principal component analysis (PCA) was performed for structuring correlation between studied samples, analysed phenolics compounds and their antihyperlipidemic properties (XLSTAT, version 2016, Addinsoft, New York, US). Diagrams were plotted using Sigmaplot software version 14.0.

3. Results and Discussion

3.1. Identification and Quantification of Phenolic Compounds in H. sabdariffa Powders

The main of phenolic compounds in powders fraction obtained by CDSp were identified and quantified by LC- / MS. Standard Compound Analyzes by LC- MS determine retention times and m / z ratios of compounds. The UV chromatogram in Figure 1 shows the separation of the different phenolic compounds extracted from the calyxes of *H. sabdariffa*. It shows the presence of seven phenolic compounds including four phenolic acids, namely protocatechic acid (m/z 153, tr: 4.46min) and chlorogenic acid (m/z 353, tr: 8.19 min), p-coumaric acid (m/z 163, tr: 14.12min) and ferulic acid (m/z 193, tr: 16.80 min); and three flavonoids namely catechin (m/z 289), rutin (m/z 603) and quercetin (m/z 301) identified at 7.32min, 18.86min and 22.62min respectively. Their contents were quantified in all CDSp fractions, unsieved powder, and ethanolic extract of H. sabdariffa (Figure 3). These results show that the majority compounds in all samples are ferulic acid (80.01mg/g DM for the fraction 212-315µm) and chlorogenic acid (73.50mg/gDM for the fraction 212-315µm), and the lowest is quercetin (1.05mg/gDM for the fraction 212-315µm). The influence of the granulometric class on the content of these phenolic compounds was observed because their concentrations vary from one fraction to another. Indeed chlorogenic acid, quercetin, protocatechuic acid and catechin are more concentrated in the fraction of particle size class [212-315µm] compared to the other fractions and unsieved powder. Compared to the ethanolic extract this fraction also shows significantly higher contents of these compounds except for protocatechic acid which is equally concentrated in both extracts. The lowest contents of these four

compounds were obtained for the fraction ≥315µm and the unsieved powder. Ferulic acid and p-coumaric acid were similarly concentrated in the <180µm and 212-315µm particle size class powder fractions and have the maximum contents of these compounds compared with the other fractions and the unscreened powder. These two fractions have significantly higher concentrations of ferulic acid than the ethanolic extract but concentrate p-coumaric acid in the same way. The lowest concentrations of these two phenolic compounds were obtained in the ≥315µm powder fraction. Rutin, on the other hand, was more concentrated in the powder fraction 180 µm compared with the other fractions and the ethanolic extract. This could be explained by the fact that grinding followed by a sieving process would induce the release of phenolic compounds with an increase in the specific surface area of the powders. They are synthesised in chloroplasts; thus, their bioaccessibility was increased after degradation of plant cells during the milling process [27]. Indeed, large particles, richer in carbohydrates and probably in fibers are expected to contain less phenolic compounds [28].

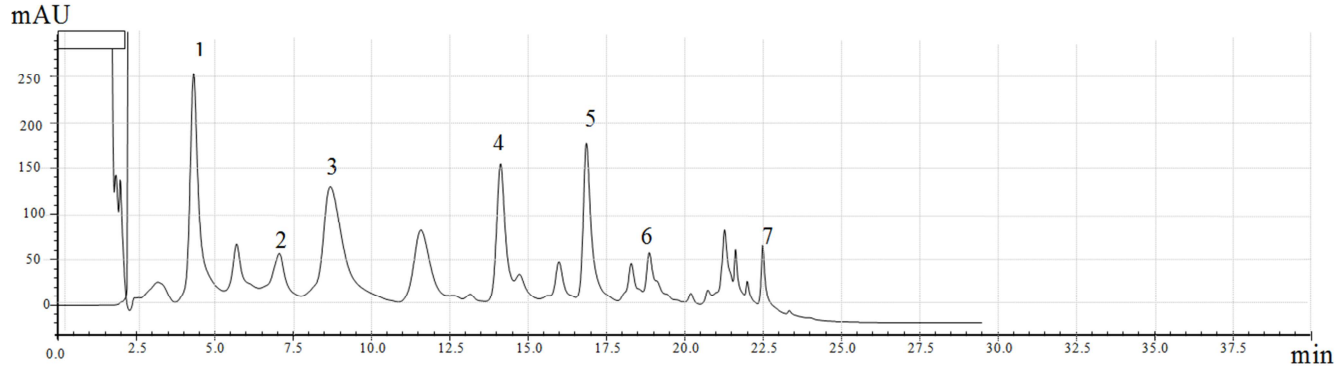
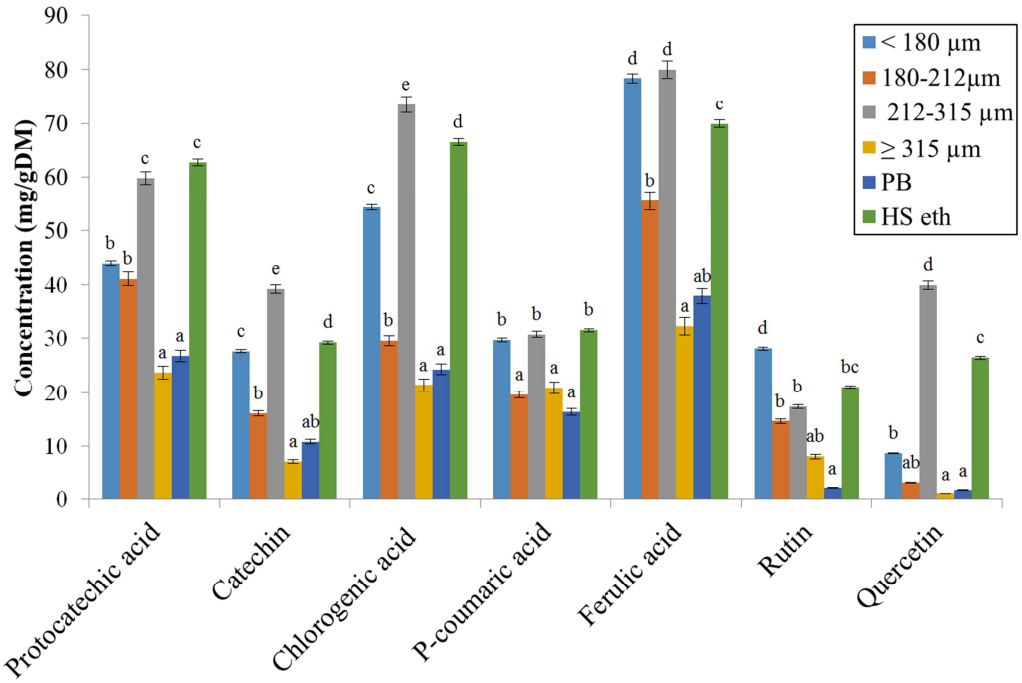


Figure 1. *Chromatogram of the unsieved powder of H. sabdariffa.*



PB: Unsieved powder HS eth: ethanolic extract. Histograms with different letters are significantly different at the p<0.05 threshold, n=3.

Figure 2. *Contents of phenolic compounds identified in powder fractions and ethanolic extract of H. sabdariffa.*

3.2. Effect of H. sabdariffa Powders (CDSp Fractions, Unsieved Powder and Ethanolic Extract) on Food Intake, Weight Gain and Fecal Lipids of Rat

Table 1 presents the effect of *H. sabdariffa* powder fractions on the weight, food intake and fecal lipids of rats subjected to the hyperlipidic diet. From these, it was found that the amount of feed consumed by the different groups of animals showed no significant difference (p<0.05). This result means that the dose chosen did not affect the palatability of the animals. However, the results obtained show that the powder samples from *H. sabdariffa* exhibited an effect on body weight in high-fat diet rats through the reduction of body weight gain and this property depend on particle size of *H. sabdariffa* powders. All

groups had body weight between 200 and 300 g in the beginning of the experiment and received similar amount of calories for the entire period. At the end of treatment, the body weight in negative control group gained more than those in normal group and others rat groups ($p<0.05$). Meanwhile, the <180 µm and 212-315µm same to the ethanolic extract treated groups (250 mg/kg) were exhibited a remarkably reduced weight gain compared to negative control group. These results allow us to confirm that the different samples of *H. sabdariffa* powder act in an effective way on the reduction of fats, hence the slowing down of weight gain in the test groups. The decrease in weight gain observed in the test groups may be due to their ability to reduce fat absorption and lipogenic enzymes and to increase fat excretion. Indeed, the lower the weight gain, the higher the amount of fecal lipids. Similar results were obtained by the work of Deli *et al*. [16] on the in vivo antioxidant activity of the powder fractions and the ethanolic extract of *H. sabdariffa* calyxes, which demonstrate that rats treated with the fraction of particle size class ≤180 µm show the lowest weight gain compared to the other fractions and to the crude powder, but it is identical to those treated with the ethanolic extract of the plant.

Table 1. *Food intake, weight gain and fecal lipids of rats fed a hyperlipidic diet at the end of antihyperlipidemic test.*

Groups	Food intake (g/day)	Weight gain (g)	Fecal Lipids (mg)
Normal control	33.03±0.31^{a}	30.23±9.15^{a}	16.28±3.36^{a}
Negative control	32.81±1.26^{a}	112.62±8.93^{d}	19.16±2.61^{a}
Positive control	32.99±1.77^{a}	32.02±5.39^{a}	100.26±1.43^{d}
F1 (<180µm)	32.81±1.86^{a}	61.72±5.47^{b}	84.05±2.65^{c}
F2 (180-212µm)	33.0±1.27^{a}	70.32±6.04^{c}	55.47±2.56^{b}
F3 (212-315µm)	33.07±1.91^{a}	62.72±6.42^{b}	85.54±3.09^{c}
F4 (≥ 315 µm)	32.94±1.85^{a}	71.18±7.81b^{c}	67.26±1.08^{bc}
Unsieved powder	32.88±0.7^{a}	82.43±5.06^{d}	60.54±3.57^{b}
Ethanolic extract	32.77±1.48^{a}	60.02±6.47^{b}	82.22±3.99^{c}

Mean ± standard deviation on the same column with different letters at exponent are significantly different at the threshold of $p<0.05$; n=3.

3.3. Effect of H. sabdariffa Powders (CDSp Fractions, Unsieved Powder and Ethanolic Extract) on Relative Mass of Organs (Organ Index)

The organ indices inform us about the evolution of the organ in relation to that of the whole organism, that is, about the toxicity of the substance used. Indeed, the variation of the organs mass is significant of toxicity, an increase of this one after consumption of a substance means that this one is toxic [29]. Table 2 shows the effect of different samples of *H. sabdariffa* powder on the relative mass of the organs of rats after four weeks of treatment. The results obtained show on the one hand that at the level of heart, kidneys and lungs, liver and testicles there is no significant difference ($p<0.05$) between the groups subjected to the treatments based on the powder fractions, the raw powder and the ethanolic extract and the normal control in the three tables. This indicates that the latter have no effect on the relative weight of the organs. These results confirm the safety of the treatments applied to the plants (CDSp fractions, and freeze dried ethanolic extract) and the non-toxicity of the dose used in our experiment. This result is in agreement with that of Ahmed *et al*. [30], which show that if the values of the organs do not present differences after a treatment, it would mean that the administered substance is not toxic. On the other hand, there is an increase of 55% in the relative mass of the liver of the negative control animals compared to the normal control animals. This may be one of the consequences of the excessive consumption of triglycerides that will be at the origin of the liver dysfunction characterized by an accumulation of fats in the liver [31]. This accumulation is at the origin of the increase in liver weight. Finally, we note that the relative mass of the testes of the positive control rats subjected to atorvastatin is significantly lower ($p<0.05$) than that of all the other groups in the different tables. It can be concluded that atorvastatin induced a decrease in testicular size of adult male albino rats. These results corroborate those obtained by Amany *et al*. [32] who demonstrate that atorvastatin causes various deleterious changes in the histological structure of adult male albino rat testes, among which the significant decrease in their sizes.

Table 2. *Organ index of rats at end of antihyperlipidemic test.*

Groups	Heart	Liver	Kidney	Lung	Testes
Normal control	0.39±0.02^{a}	3.23±0.30^{a}	0.68±0.02^{a}	0.71±0.11^{a}	1.21±0.18^{b}
Negative control	0.40±0.05^{a}	4.77±0.25^{b}	0.67±0.09^{a}	0.73±0.15^{a}	1.23±0.29^{b}
Positive control	0.39±0.01^{a}	3.28±0.23^{a}	0.69±0.06^{a}	0.70±0.19^{a}	0.59±0.20^{a}
F1 (<180µm)	0.41±0.04^{a}	3.23±0.14^{a}	0.68±0.02^{a}	0.76±0.13^{a}	1.29±0.25^{b}
F2 (180-212 µm)	0.38±0.03^{a}	3.22±0.21^{a}	0.66±0.06^{a}	0.72±0.14^{a}	1.24±0.27^{b}
F3 (212-315µm)	0.41±0.04^{a}	3.32± 0.20^{a}	0.64±0.04^{a}	0.78±0.17^{a}	1.22±0.2^{b}
F4 (≥ 315 µm)	0.36±0.03^{a}	3.24±0.13^{a}	0.70±0.08^{a}	0.74±0.16^{a}	1.29±0.16^{b}
Unsieved powder	0.41±0.04^{a}	3.27±0.16^{a}	0.68±0.07^{a}	0.75±0.19^{a}	1.25±0.22^{b}
Ethanolic extract	0.37±0.02^{a}	3.25±0.11^{a}	0.65±0.07^{a}	0.77±0.2^{a}	1.27±0.15^{b}

Mean ± standard deviation on the same column with different letters at exponent are significantly different at the threshold of $p<0.05$; n=5.

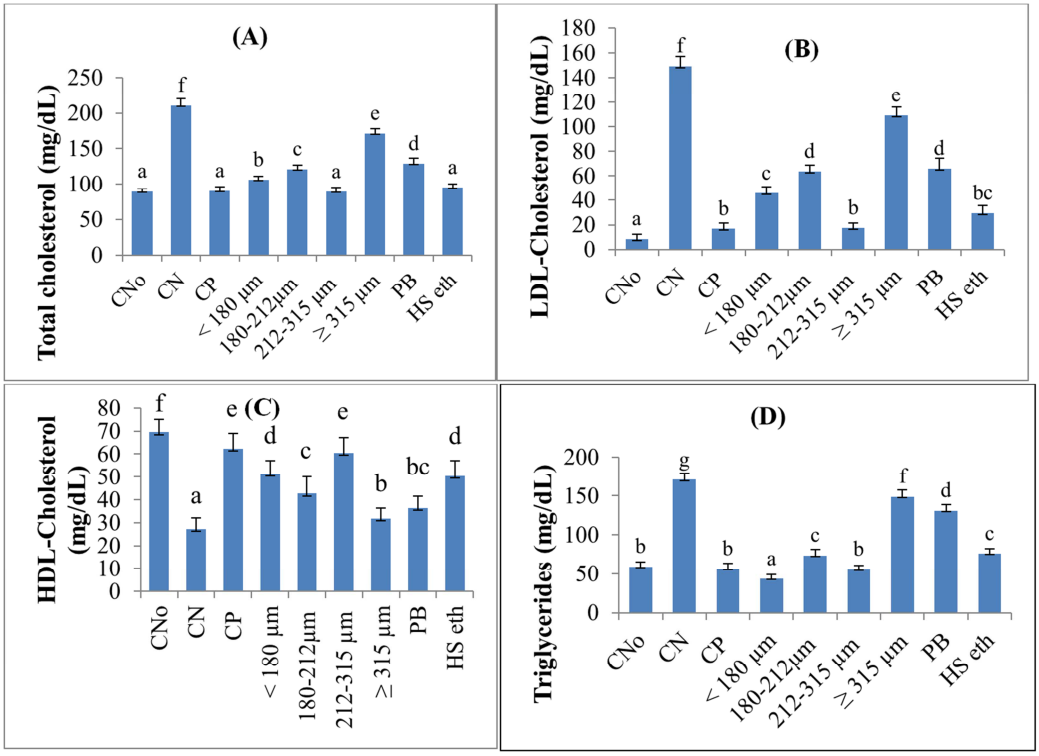


Figure 3. *(A-D) A Serum levels of CT, B) LDL-C, C) HDL-C, D) TG of rats of H. sabdariffa powders (CDSp fractions, unsieved powder and freeze-dried ethanolic extract) at the end of experimentation.*

CNo: normal control; CN: negative control; CP: positive control; PB: crude powder; HS eth: ethanolic extract of H. sabdariffa; n=5. For each parameter, Error bars represent standard deviation, histograms with different letters are significantly different at the $p<0.05$ threshold.

3.4. Effect of H. sabdariffa Powders (CDSp Fractions, Unsieved Powder and Freeze-Dried Ethanolic Extract) on the Lipidemia Parameters of Rats

Serum levels of total cholesterol TC, LDL-C, HDL-C and TG are parameters of the lipid profile without which a correct interpretation would not be made. LDL-C is the fraction of cholesterol that exerts an atherogenic action and is therefore considered an important risk factor for cardiovascular disease [24]. While HDL-C is the anti-risk marker for cardiovascular disease, its decrease increases the risk of CVD. Hypertriglyceridemia is an independent risk factor for CVD. However, the reduction of TG leads to a reduction in atherogenicity because the presence of TG causes a delay in the catabolism of triglyceride-rich lipoproteins and induces the appearance of dense and more oxidizable LDL [34]. Figure 3 shows the effect of different *H. sabdariffa* powder samples on the lipid profile parameters of rats. Animals subjected to the different samples of *H. sabdariffa* calyxes have significantly lower serum levels of total TC, LDL-C and TG ($p<0.05$) compared to those of the negative control. While their serum HDL-C levels are significantly higher ($p<0.05$) than those of the negative control. However, animals treated with the 212-315µm and 180µm particle size class fractions had the best lipid parameters with significantly lower serum TC, LDL-C and TG levels and significantly higher serum HDL-C levels compared to those of the rats treated with the other powder fractions obtained by CDSp, and the crude powder. The lowest activity was observed in rats subjected to the fraction of particle size class ≥315 µm and crude powder with the highest levels of total and LDL-C and TG and very low serum levels of HDL-C. The comparison of the granulometric class (212-315µm) with the best antihyperlipidemic properties to the ethanolic extract shows that the rats treated with it have TC levels that are not significantly different from those treated with the ethanolic extract. While the levels of LDL-cholesterol and triglycerides of these rats are significantly lower compared to those treated with ethanolic extract. However at the level of HDL-C, the rats subjected to ethanolic extract have a higher level of HDL-Cl compared to those treated with the fraction of the 212-315µm particle size class.

The reduction of blood levels of TC, LDL-C and TG as well as the increase of HDL-cholesterol observed in the groups treated with the powder fractions obtained by CDSp, crude powder and ethanolic extract of *H. sabdariffa* compared to the negative control is explained by the richness of this plant in phenolic compounds as observed in their characterisation. Numerous studies have demonstrated a correlation between the consumption of the latter and a decrease in cholesterol. The example of quercetin from black tea, which inhibits cholesterol absorption by disrupting micelle formation [35]. The hypocholesterolemic effect *of Lonicera caerulea* berry extract rich in catechin and chlorogenic acid has been demonstrated by the work of Rabie *et al*. [36]. Indeed, these phenolic compounds can act by inhibiting HMG-CoA reductase, a key

enzyme in endogenous cholesterol synthesis at the cellular level. They can also catalyze the conversion of HMG-CoA to mevalonate which is an early step limiting cholesterol synthesis. Decreased cholesterol synthesis stimulates LDL-C receptor gene expression by lifting the negative feedback control exerted by intracellular cholesterol. Thus, the increase in LDL-C receptor synthesis leads to an increase in plasma LDL-C uptake and catabolism by cells, thus decreasing LDL levels and increasing HDL-C [37]. At the level of TG, these phenolic compounds have the ability to decrease the absorption of lipids by inhibiting pancreatic lipase which is the most important enzyme for the efficient digestion of the latter [38-40]. They can act directly on the site of the active enzyme or, indirectly, by increasing the size of the lipid droplets, thereby reducing the accessibility of the substrate to the enzyme. This effect is likely to limit the intestinal absorption of triglycerides. It is also observed that the rate of the different parameters measured varies from one granulometric class to another. This variation can be explained by the fact that the distribution of phenolic compounds in the different fractions varies from one particle size class to another.

3.5. Atherogenicity Ratios

Table 3 shows the TC/HDL-C and LDL-C/HDL-C ratios of rats subjected to different *H. sabdariffa* powder samples. In general, the TC/HDL-C and LDL-C/HDL-C ratios of the animals that received the CDSp powder fractions, the unsieved powder, and the ethanolic extract are lower than those of the negative control. These animals have values within the normal range, in contrast to those of the negative control, which have values above the normal range. These results confirm on the one hand that all CDSp powder fractions have antihyperlipidemic properties because they decrease the level of cholesterol in the blood, thus protecting the organism from cardiovascular diseases with normal atherogenic indices. On the other hand, these results also confirm that the 212-315µm fraction is the one with superior antihyperlipidemic properties than the other fractions and their unscreened powders because their atherogenic indices are lower. However, the atherogenic indices of this powder fraction are lower than those of the ethanolic extract but do not show a significant difference for the TC/HDL-C ratio. This suggests that the CDSp improved the protective effect of *H. sabdariffa* against hyperlipidemia better than ethanol extraction.

Table 3. *TC/HDL-C and LDL-C/HDL-C ratios of rats.*

Groups	TC/HDL-C	LDL-C/HDL-C
Normal control	2.27 ± 0.10^{bcd}	0.62 ± 0.11^{a}
Negative control	7.75 ± 0.72^{f}	5.83 ± 0.96^{d}
Positive control	1.66 ± 0.10^{ab}	0.49 ± 0.08^{a}
F1 (<180µm)	2.06 ± 0.12^{abc}	0.89 ± 0.11^{ab}
F2 (180-212 µm)	2.82 ± 0.18^{d}	1.50 ± 0.20^{b}
F3 (212-315µm)	1.47 ± 0.17^{a}	0.29 ± 0.18^{a}
F4 (≥ 315 µm)	5.43 ± 0.71^{e}	3.38 ± 0.61^{c}
Unsieved powder	2.60 ± 0.56^{cd}	0.96 ± 0.39^{ab}
Ethanolic extract	1.88 ± 0.12^{ab}	0.58 ± 0.13^{a}

3.6. Effect of H. sabdariffa Powders (CDSp Fractions, Unsieved Powder and Freeze-Dried Ethanolic Extract) on Creatinine and Transaminases (ALAT and ASAT).

(ASAT) and ALAT) are the important markers of liver function, a critical organ in maintaining cholesterol homeostasis. The assessment of these two parameters helps to determine possible abnormalities caused by hyperlipidemia [41]. ALAT is a liver-specific enzyme, making it an important and sensitive indicator of hepatotoxicity. TSA is also an indicator of hepatocyte destruction although in addition to the liver, it is found in the heart, skeletal muscle, lung and kidney. Serum levels of ALAT and ASAT rise rapidly when the liver is damaged [41]. Table 4 show that the serum ALAT and ASAT levels of the negative control rats fed only a hyperlipidic diet are higher ($p<0.05$) than those of the normal control. However, the ALAT and ASAT levels of the groups subjected to the powder fractions obtained by CDSp are lower than those of the negative control. These results show that the powders have a hepatoprotective effect because they protected the liver from the damage caused by the hyperlipidic diet. But, the highest hepatoprotective activity is observed in rats treated with the 212-315µm and 180µm powder fractions. It does not show any significant difference compared to the activity of the ethanolic extract. Thus, these fractions protect the liver of rats from hyperlipidic diet-related damage as much as the ethanolic extract. These results are in line with those obtained by Nurkhasanah and Muhammad [40] who demonstrated the hepatoprotective effect of the ethanolic extract of *H. sabdariffa* on rats.

Creatinine represents the major biological marker of renal impairment. It is a product of the non-enzymatic degradation of creatine, a protein compound contained in muscle tissue, and is strictly eliminated by the kidneys. Thus, the change in their serum level is a reliable indicator of disorders concerning glomerular filtration and renal dysfunction, a factor leading to CVD. This study shows that the creatinine levels of the groups of animals subjected to the different samples of *H. sabdariffa* powder are identical to those of the normal control but significantly lower than those of the negative control. These results demonstrate the nephroprotective effect of the powder fractions obtained by CDSp.

Table 4. *Transaminase and creatinine levels in treated rats at the end of experimentation.*

Groups	ASAT (UI/L)	ALAT (UI/L)	Creatinine (mg/dL)
Normal control	97.11 ± 4.68^{a}	60.66 ± 2.98^{a}	17.99 ± 0.91^{a}
Negative control	135.22 ± 2.29^{b}	83.13 ± 4.15^{c}	29.17 ± 0.14^{b}
Positive control	95.90 ± 4.11^{a}	62.46 ± 3.47^{a}	16.01 ± 0.39^{a}
F1 (<180µm)	98.02 ± 2.44^{a}	60.05 ± 2.27^{a}	15.86 ± 0.75^{a}
F2 (180-212 µm)	100.52 ± 6.60^{a}	72.09 ± 3.26^{b}	19.01 ± 0.32^{a}
F3 (212-315µm)	96.50 ± 1.37^{b}	61.06 ± 2.55^{a}	17.45 ± 0.44^{a}
F4 (≥ 315 µm)	105.50 ± 3.23^{ab}	70.21 ± 4.77^{b}	17.93 ± 0.41^{a}
Unsieved powder	102.12 ± 2.44^{ab}	67.06 ± 4.21^{ab}	18.15 ± 0.29^{a}
Ethanolic extract	97.89 ± 3.18^{a}	63.99 ± 4.55^{a}	16.15 ± 0.52^{a}

Mean ± standard deviation on the same column with different letters at exponent are significantly different at the threshold of $p<0.05$; n=5

3.7. Correlation and PCA

Figure 4 shows the PCA of antihyperlipidemic properties and phenolic content of the powder fractions and freeze-dried ethanolic extract of *H. sabdariffa*. The powder samples, phenolic compound content and resulting antihyperlipidemic properties were projected into a single system. The obtained PCA explains 90.57% of the total variation that exists in this system with a contribution of 82.67% for the F1 axis and 7.90% for the F2 axis. It is observed that the content of phenolic compounds (quercetin, catechin, rutin, pprotocatechic acid, p-coumaric acid, ferulic acid and chlorogenic acid) is strongly positively correlated with the serum level of HDL-C, while a negative correlation was observed with the serum levels of LDL-C, total cholestaeol, triglycerides, the ratios CT/HDL-C and HDL-C/ LDL-C and transaminases (Asat and Alat). Indeed, as shown in Figure 4, the phenolic compounds allowed the increase of serum HDL-C and the decrease of serum levels of LDL-C, total cholesterol, triglycerides, CT/HDL-C and HDL-C/ LDL-C ratios and transaminases in the <180µm, 212-315µm powder fractions and the ethanolic extract. The powder fractions of particle size class 180-212µm and ≥315µm as well as the unsieved powder are significantly less rich in phenolic compounds.

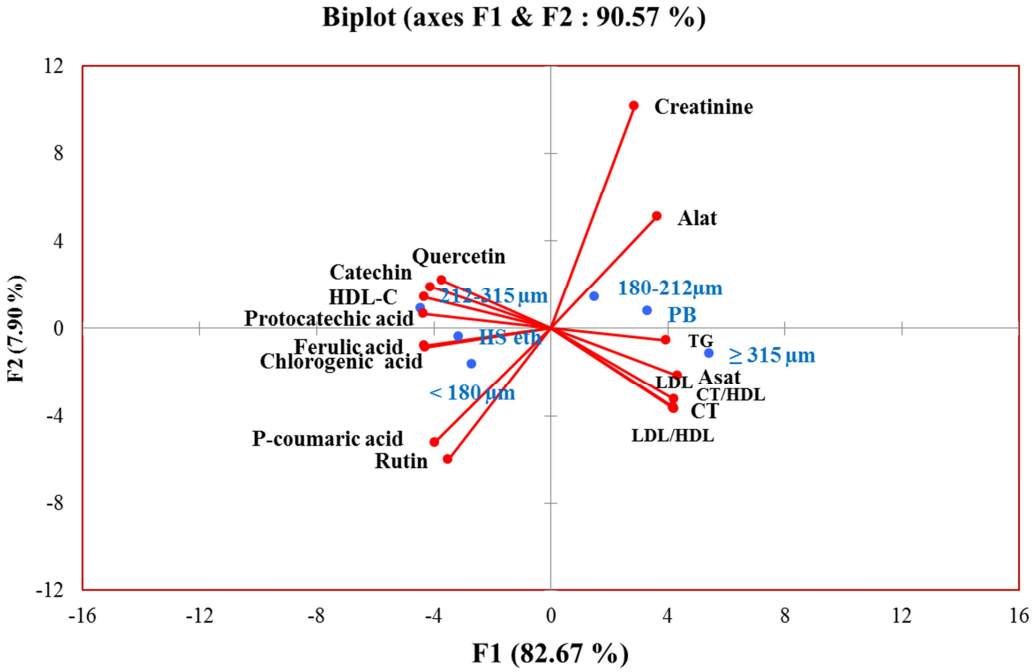


Figure 4. *Principal components Biplot (axes F1 & F2: 90.57%) analysis of phenolic compound contents and antihyperlipidemic properties of investigated H. sabdariffa powders (CDSp fractions, unsieved powder, and freeze-dried ethanolic extract).*

4. Conclusion

Phenolic characterisation and antihyperlipidemic activity of *H. sabdariffa* calyx powder samples obtained CDSp was investigated in this study and compared to ethanolic extraction. It can be concluded that the CDSp applied to *H. sabdariffa* was successful in improving the phenolic compound content of the different powder fractions. However, the fractions of particle size class <180µm and 212-315µm were the ones with higher phenolic compound contents and higher preventive activity against hyperlipidemia compared to the other particles. The ethanolic extract was also rich in phenolic compounds but the 212-315µm powder fraction had higher contents of chlorogenic acid, quercetin, catechin and ferrulic acid. In terms of antihyperlipidemic activity, the 212-315µm fraction generally had a higher activity than the ethanolic extract. Thus, CDSp is useful and effective to improve the content of phenolic compounds in the *H. sabdariffa* powder and allow by ricochet the improvement of their antihyperlipidemic properties, which will allow managing cardiovascular diseases.

Conflicts of Interest

The authors declare that they have no competing interests.

Compliance with Ethics Requirements

Research approval was obtained from the Cameroonian National Ethics Committee Ref. No. FWIRD00001954.

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