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Phenolic characterization and lipid lowering properties of powder fractions and ethanolic extract of *Adansonia digitata pulp.*

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Key words: Phenolic compounds; antihyperlipidemic activity; *Adansonia digitata* pulp; powder fraction; lyophilized ethanolic extract.

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Abstract

This study aimed to evaluate the effect of controlled differential sieving treatment on the phenolic content and antihyperlipidemic activity of *A. digitata* pulp and compared it to the effect of ethanolic extract. For this purpose, powders with particle sizes ranging from 20 to 100 µm and above were produced and analyzed. As a first step, 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). Then, 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 lyophilized ethanolic extract. LC-MS analysis identified four compounds (quercetin, rutin, ferrulic acid and cafeic acid) in the different sample of *A. digitata* pulp powder. The granulometric class <50µm showed the best concentration, higher than those of the other granulometric class and crude powder. This fraction also showed content of phenolic compounds similar to those of ethanolic extract except cafeic acid which was more concentrated in the ethanolic extract. The antihyperlipidemic activity were significantly influenced (P <0.05) by particle sizes. The best activity was obtained from the <50µm µm fraction which was significantly (P <0.05) higher than the other fractions and the unsieved powder to improve the lipid parameters (TC, LDL-C, HDL-C and TG), transaminases (ALAT and ASAT) and creatinine. It showed similar activity to the ethanolic extract with the exception of LDL-C which is lower in this fraction. Controlled differential sieving process 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.

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Introduction

Adansonia digitata is a plant belonging to the Bombacaceae family and more commonly known as Baobab (French) and Monkey Bread Tree (English). Over the years, it has attracted the attention of several researchers, in particular for its pulp which has many nutraceutical and medicinal properties (Acham *et al.*, 2020; Alrasheid *et al.*, 2019). Among these properties we have its ability to fight against hyperlipidemia, which is one of the major risk factors for cardiovascular diseases which are today the leading cause of death in the world (WHO, 2017). Thus, these properties attributed to the pulp of *A. digitata* are due to the bioactive molecules it abounds (Muhammad *et al.*, 2016; Ngatchic *et al.*, 2020). However, to be valorized, these active compounds are generally extracted from the original plant matrix. The extraction of active ingredients from plant matter, such as phenolic compounds which are currently attracting growing interest because of their role as natural antioxidants, is therefore a crucial step: it determines the quantity and nature of the molecules extracted, of which depend on the expected biological properties (Ameer *et al.*, 2017).

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 (Monroy *et al*, 2016). 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 ingredients (Leonardo *et al.*, 2013; Miron *et al.*, 2010).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 (CDS) process, which reduces these collateral effects, while ensuring a safe and high quality natural extract. CDS 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 (Baudelaire, 2013). The efficiency of this extraction process has been demonstrated by several studies (Becker *et al.*, 2016; Deli *et al.*, 2020; Zaiter *et al.*, 2017). 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 *A. digitata* compared to ethanol extraction.

Materials and methods

Reagents and plant sample

HPLC grade acetonitrile, water, methanol, ethanol and the standards were purchased from Sigma-Aldrich. The measurement kits for the biochemical parameters were obtained from ENSAI. The plant material used in the present study consists of dried pulp of *A. digitata*. This plant material was purchased from the local market in the Adamaoua region of Cameroon. They were cleaned by hand to remove foreign bodies before being ground for powder production.

Plant grinding and controlled differential sieving process

Grinding was carried out using a ZM 200 ultra-centrifugal electric mill equipped with a 99 mm 24-tooth rotor and a 1 mm trapezoidal hole sieve. The powder obtained was then sieved according to the procedure previously described by Deli *et al*, 2019a and Deli *et al*, 2019b. For this purpose, 100 g of powder were passed through sieving columns using an Analysette 3 Spartan apparatus (Fritsch) to obtain fractionated powders.

The vibration amplitude of the sieve shaker was set at 0.5 mm for 10 min. Thus, the powder samples were classified into four main categories as follows: <50, 50-100, and ≥100µm. The unsieved powder was taken as a control, and they were packed in plastic bags and stored at 10°C until analysis.

Production of lyophilized ethanolic extract powder

Unsieved powder was mixed with ethanol in the ratio of 1/10 (w/v) and stand under magnetic stirring (Variomag Poly) for 24 hr at 18°C. The homogenate obtained was then filtrated with whatman No 1 filter paper (pore size 12-15lm) 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.

LC-MS analyses

Extraction of phenolic compounds

For the preparation of the extracts, 2g of each powder fraction was mixed in 20ml of water-methanol mixture 30:70 (v/v) by stirring (300rpm) at room temperature of the laboratory (18 ± 2°C) for 24h. 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 (Cuji'c *et al*., 2015). 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.

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 × 4.6mm. C18 inverse phase Gemini column (Phenomenex, Torrance, CA, USA) with a particle size of 3µm 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 20µl 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; 15-18min 20-25; 18-22min, 25-60; 22-25min of 60:60; 25-28min, 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 at 250°C. Double detection was ensured by a photodiode array detector (PDA) in the 200-400nm wavelength range and mass spectrometry (MS). MS settings were as follows: negative mode electrospray source (ESI), 1.5L/min nebulization gas flow, 15L/min drying gas flow, 300°C heat block temperature, 250°C desolvation line temperature, -4kV probe voltage, and selected-ion monitoring (SIM) acquisition.

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: 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); Ferrulic acid (R^2 = 0.995; LOD= 2.2 ppm; LOQ= 7.4) and Rutin (R^2 = 0.998; LOD= 0.3 ppm; LOQ= 0.9 ppm). Results were expressed in mg of standard per 100 grams of dry matter.

Animals and induction of hyperlipidemia

The complete experiment was carried out using 40 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.* (2008). 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 (Hamlat *et al.*, 2008; Ngatchic *et al.*, 2016).

Experimental procedure

During 28 days of experimentation, the animals were fed and treated simultaneously based on the different powder fractions obtained by CDS process, unsieved powder and lyophilized ethanolic extract of *A. digitata*. 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 10mL/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 8 groups of 5 rats: normal control group, negative control group, positive control group, and 5 experimental groups who receiving powder fractions of <50µm, 50-100µm and ≥100µm, 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 1h before 10 mL/kg of aqueous suspension of *A. digitata* powder for the experimental groups, Atorvastatin (10mg/kg) for the positive control group, and distilled water for the negative.

Measurement of some physical parameters of the rats treated by A. digitata powders fractions (CDS), unsieved powder and lyophilized ethanolic extract

Measurement of food intake and weight gain

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).

$$\Delta P = P_f - P_i \quad \text{With} \quad \begin{array}{l} \Delta P : gain\ (g) \\ P_i : initial\ weight\ (g) \\ P_f : final\ weight\ (g) \end{array}$$

Measurement of faecal lipid

The lipid content of feces was evaluated after extraction with Soxhlet by hexane according to the Russian method described by Bourely (1982). The extraction is based on the differential solubility of lipids in an organic solvent (hexane or petroleum ether) at high temperature. 2g of dried faeces were introduced in filter paper previously dried in the oven 105°C during 1h 30min and weighed. The whole was placed in the extractor of the soxhlet. The extractor was then mounted on a flask containing 200mL of hexane placed in the flask heater. Once the soxhlet cooler was installed, the valve opened and the flask heater turned on. The whole was heated and the extraction was performed for about 10 hours until decoloration of the packed samples contained in the extractor. At the end of the extraction, the system was stopped. The bags were removed and put in the oven at 105°C for 1 hour and weighed. The lipid content of the feces was calculated by the following formula:

$$TL = \left(\frac{M1-M2}{M1-M0}\right) * 100 \quad \text{with} \quad \begin{array}{l} TL : lipids\ content\ (mg) \\ M0 : \text{mass of the empty filter paper bag}\ (mg) \\ M1 : paper\ mass + sample\ mass + oil\ (mg) \\ M2 : paper\ mass + sample\ mass - oil\ (mg) \end{array}$$

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 3000 rpm 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). This study was carried out with approval from the Cameroonian National Ethics Committee Ref. No FWIRD00001954.

$$OI = \left(\frac{M_{org}}{M_{cor}}\right) * 100 \quad \text{with} \quad \begin{array}{l} OI : organ\ index \\ M_{Org} : organ\ mass\ (g) \\ M_{cor} : body\ mass\ (g) \end{array}$$

Determination of lipid profile parameters

Total cholesterol (TC)

TC was determined according to the enzymatic method described by Naito *et al.* (1984). 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 10min at 25°C, the absorbance of the sample was read against the reagent blank at 505nm using a spectrophotometer. The total cholesterol concentration will be determined by the following formula:

$$TC = \left(\frac{D.O\ échantillon}{D.O\ standard}\right) * 200 \quad \text{with} \quad \begin{array}{l} TC : total\ cholesterol\ (mg/dL) \\ D.O : light\ density \end{array}$$

HDL-cholesterol (HDL-C)

HDL-C was determined by the enzymatic method described by Gordon (1977). 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 500µL of precipitation reagent were added to the centrifuge tubes. After homogenization, the tubes were allowed to stand for 10min at room temperature, then centrifuged at 4000rpm for 10min and the supernatant was collected. In the assay tubes, 100µL 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 10min, the optical density was read at 500nm against the blank. The HDL cholesterol concentration of the sample is calculated according to the following formula:

$$HDL\text{-}C = \left(\frac{D.O\ échantillon}{D.O\ standard}\right) * 175 \quad \text{with} \quad \begin{array}{l} HDL - C : cholestérol\ HDL\ (mg/dL) \\ D.O : light\ density \end{array}$$

Triglycerides (TG)

TG were determined according to the method described by Fossati *et al.* (1982) with some modifications. This method is based on the principle that under the action of lipoprotein lipases, glycerol produced by enzymatic hydrolysis of triglycerides is phosphorylated by ATP to produce glycerol-3-phosphate and ADP through a reaction catalysed by glycerolkinase. Glycerol-3-phosphate oxidase then catalyses the oxidation of glycerol-3-phosphate to produce dihydroxyacetone-3-phosphate and H2O2. 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 triglycerides present in the sample. In the assay tubes, 10µL of serum and 10L of standard were introduced respectively. In these tubes, 1000µL 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 triglycerides in serum is given by the following relation:

$$TG = \left(\frac{D.O\ échantillon}{D.O\ standard}\right) * 250 \quad \text{with} \quad \begin{array}{l} TG : cholestérol\ total\ (mg/dL) \\ D.O : optical\ density \end{array}$$

LDL-cholesterol (LDL-C)

LDL-C concentration was calculated from total cholesterol concentration, HDL-C concentration, and triglyceride concentration according to the formula describe by Friedewald *et al.* (1972):

$$\text{LDL-C} = \text{TC} - \text{HDL-C}\left(\frac{\text{TG}}{5}\right) \quad \text{with } LDL-C: \text{cholestérol LDL } (mg/dL)$$

Determination of transaminases (ALAT and ASAT) and Creatinine

Creatinine

Creatinine was determined according to the method of Henry (1974), 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 1mL of plasma. The resulting solution was mixed, centrifuged at 2500rpm for 10 min and the supernatant was collected. In the white and standard tubes was introduced 0.5 mL of trichloroacetic acid solution, then 1mL of supernatant, 0.5 mL, 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 1mL 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:

$$\text{Creatinine sample} = 2 \times \frac{\text{D.O.sample}}{2 \times \text{D.O.standard}} \text{ (mg/dL)}$$

ALAT levels

The levels of alanine and aspartate aminotransferase were determined using the Randox kit according to the method of Reitma *et al.* (1957). The principle of this method is that ALAT transfers the amino group of alanine to α-oxoglutarate to form pyruvate and L-glutamate. With 2,4-dinitrophenylhydrazine, pyruvate forms pyruvate hydrazone. ALAT is determined by following the concentration of pyruvate hydrazone. In the tubes of assay and blank, are introduced respectively 0.1mL of sample and 0.1mL of distilled water, then we added in each of these tubes, 0.5mL of buffer solution. The obtained solution is mixed and incubated at 37°C for 30 min. After incubation we also introduced into these tubes, 0.5mL of 2,4-dinitrophenylhydrazine solution the mixture was made and the solutions incubated at 25°C for 20min. In the obtained solutions, 5mL of sodium hydroxide solution was introduced and the absorbance of the sample against the blank was read after 5min 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 5min, and then 0.02mL of serum was added to the test tubes. After incubation at 37°C for 1h, 0.1mL of staining reagent was added. The tubes were left at room temperature for 20min, and then the reaction was stopped by adding 1mL of NaOH. The optical densities were read at 505nm against the white tube.

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 phenolic compounds and their antihyperlipidemic properties (XLSTAT, version 2016, Addinsoft, New York, US). Diagrams were plotted using Sigmaplot software version 14.0.

Results

Identification and quantification of phenolic compounds in A. digitata.

The UV chromatogram shown in figure 1 presents the

profile of phenolic compounds extracted from the pulp of *A. digitata*. It shows the presence of four phenolic compounds including two phenolic acids, namely caffeic acid (m/z 179) and ferulic acid (m/z 193) identified respectively at retention times of 9.33min and 16.50 min. Two flavonoids, rutin (m/z 609) and quercetin (m/z 301) were also identified at 18.86 min and 22.92 min respectively. The contents of these compounds were quantified in the different granulometric classes of powder, unsieved powder, and ethanolic extract of *A. digitata* (figure 2), and it appears that the majority compound in the different extracts is caffeic acid (45.83 mg/100gDM for the fraction <50 um) and the lowest is rutin (3.73 mg/100gDM for the unsieved power). However, it can be seen that the contents of these compounds vary from one granulometric class to another. They were more concentrated in the powder fraction <50 um compared to the other fractions and to the unsieved powder. However, compared to the ethanolic extract there was no significant difference for rutin and quercetin but the ferrulic acid content is higher in the fraction <50µm (19.06 mg/100gDM) compared to the ethanolic extract (11.02 mg/100gDM). While, ethanolic extract concentrates caffeic acid (56.42 mg/100gDM) the best compared to the other fractions. The lowest contents were obtained for the fraction ≥100 µm and the unsieved powder.

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	32.06±1.71^{a}	17.23±9.15^{a}	12.28±3.36^{a}
Negative control	33.86±2.26^{a}	99.62±8.93^{d}	14.16±2.61^{a}
Positive control	31.98±1.77^{a}	21.02±5.39^{a}	105.26±1.43^{d}
F1(<50µm)	32.56±1.27^{a}	62.72±5.47^{b}	84.05±2.65^{c}
F2(50-100µm)	32.07±1.11^{a}	63.32±6.04^{b}	82.47±2.56^{c}
F3(≥ 100µm)	31.94±1.45^{a}	73.72±8.42^{c}	61.54±3.09^{b}
Unsieved powder	32.88±0.7^{a}	72.18±7.81b^{c}	60.26±1.08^{b}
Ethanolic extract	33.77±1.22^{a}	59.43±5.06^{b}	80.54±3.57^{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.

Effect of A. digitata powders fractions, unsieved powder and ethanolic extract on food intake, weight gain and fecal lipids of rat.

The effect of the *A. digitata* powder fractions, unsieved powder and ethanolic extract on the weight, food intake and faecal lipids of rats is presented in Table 1. Statistical analysis first reveals that the amount of food consumed by normal and negative controls is not significantly different. However, the opposite is observed for weight gain.

In fact, the weight gain of the rats on the hyperlipidic diet was greater than that of the rats on the normal diet. In the animals subjected to the different *A. digitata* treatments, the weight gain of the different groups was significantly higher than that of the positive control (21.02±5.39g). On the other hand, those who received the finest powder fractions, especially those with a particle size class between <50µm (59.43±5.06g) and 50-100µm (62.72±5.47g), showed a significantly lower weight gain ($p<0.05$) than the animals subjected to the other fractions and the raw powder. However, there was no significant difference between these two groups (<50 µm and 50-100µm) and the group of animals given the ethanolic extract (63.32±6.04g). Finally, it is observed that the lower the weight gain, the higher the amount of lipids excreted in the feces. This excretion is higher for the groups of rats subjected to the <50 µm and 50-100 µm fractions.

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

Groups	Heart	Liver	Kidney	Lung	Testes
Normal control	0.37±0.04^{a}	3.23±0.31^{a}	0.70±0.09^{a}	0.70±0.11^{a}	1.08±0.28^{b}
Negative control	0.39±0.07^{a}	4.59±0.29^{b}	0.68±0.07^{a}	0.71±0.12^{a}	1.03±0.19^{b}
Positive control	0.35±0.03^{a}	3.18±0.23^{a}	0.65±0.06^{a}	0.73±0.17^{a}	0.65±0.10^{a}
F1 (<50µm)	0.38±0.06^{a}	3.23±0.14^{a}	0.71±0.02^{a}	0.76±0.13^{a}	1.08±0.15^{b}
F2 (50-100µm)	0.33±0.03^{a}	3.22±0.22^{a}	0.66±0.06^{a}	0.72±0.14^{a}	1.03±0.27^{b}
F3 (≥ 100µm)	0.32±0.05^{a}	3.24±0.13^{a}	0.70±0.08^{a}	0.74±0.16^{a}	1.09±0.16^{b}
Unsieved powder	0.37±0.04^{a}	3.27±0.16^{a}	0.67±0.07^{a}	0.73±0.19^{a}	1.04±0.21^{b}
Ethanolic extract	0.34±0.01^{a}	3.20±0.21^{a}	0.65±0.02^{a}	0.75±0.1^{a}	1.06±0.14^{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.

Effect on relative mass of organs (organ index)

Table 2 shows the effect of different samples of *A. digitata* pulp powder on the relative organ mass of rats after four weeks of treatment. The results obtained show on the one hand that in the heart, kidneys and lungs, liver and testes there is no significant difference ($p<0.05$) between the groups subjected to the treatments with the powder fractions, the raw powder and the ethanolic extract and the normal control. On the other hand, there was a 58% increase in the relative liver mass of the negative control animals compared to the normal control animals. Finally, the relative mass of the testis of the positive control rats subjected to atorvastatin was significantly lower ($p<0.05$) than that of all other groups.

Table 3. Transaminase and creatinine levels in treated rats at the end of experimentation.

Groups	ASAT (UI/L)	ALAT (UI/L)	Creatinine (mg/dL)
Normal control	90.99±1.99^{a}	50.66±2.98^{a}	18.01±0.14^{a}
Negative control	128.28±2.55^{d}	83.23±4.16^{c}	30.11±0.88^{b}
Positive control	94.90±4.77^{b}	53.46±4.46^{a}	20.21±0.39^{a}
F1 (<50µm)	99.99±2.11^{c}	64.05±2.22^{b}	19.86±0.25^{a}
F2 (50-100µm)	101.46±5.90^{c}	68.09±2.26^{b}	19.01±0.12^{a}
F4 (≥ 100µm)	100.29±4.77^{c}	67.06±2.55^{b}	21.45±0.34^{a}
Unsieved powder	102.02±3.55^{c}	65.21±5.27^{b}	20.13±0.49^{a}
Ethanolic extract	100.01±4.01^{c}	63.02±4.33^{b}	18.99±0.84^{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.

Effect of A. digitata powders (fractions, unsieved powder and ethanolic extract) on the lipidemia parameters of rats

The group of animals subjected to sample of *A. digitata* pulp powder (figure 3) had significantly lower serum total and LDL-C levels than the negative control (171.39mg/dL for TC and 119.07mg/dL for LDL-C). However, animals treated with the 50 µm particle size fraction (109.4mg/dL for TC 49.17mg/dL for LDL-C) showed significantly lower serum total and LDL-C levels than rats treated with the other fractions as well as the crude powder. The highest levels were obtained in rats treated with the crude pulp powder (136.27mg/dL for TC and 98.83mg/dL for LDL-C). It is also noted that the 50µm powder fraction group had a total cholesterol level that was not significantly different from that of the ethanolic

powder extract group (115.4mg/dL). While their LDL cholesterol level is significantly lower than that of the animals treated with ethanolic extract (56.07mg/dL). It is also observed that the serum HDL-C level of the groups treated with the different powder fractions is higher ($p<0.05$) than that of the negative control (21.9mg/dL). However, the animals treated with the <50µm particle size fraction (44.51mg/dL) showed significantly higher HDL-C levels than the rats treated with the other fractions as well as the raw powder. However, the group treated with ethanolic extract (44.11mg/dL) had higher HDL-C levels compared to the groups treated with the fractions except for the 50µm fraction where the difference was not significant. The lowest level was observed in the rats treated with the unsieved powder (26.18mg/dL).

In terms of serum TG levels, these groups had levels that were lower ($p<0.05$) than the negative control (149.97mg/dL). All groups treated with the powder fractions have triglyceride levels that are not significantly different from those of the ethanolic extract and the crude powder except for the 50-100µm fraction (82.85mg/dL) which has a higher level.

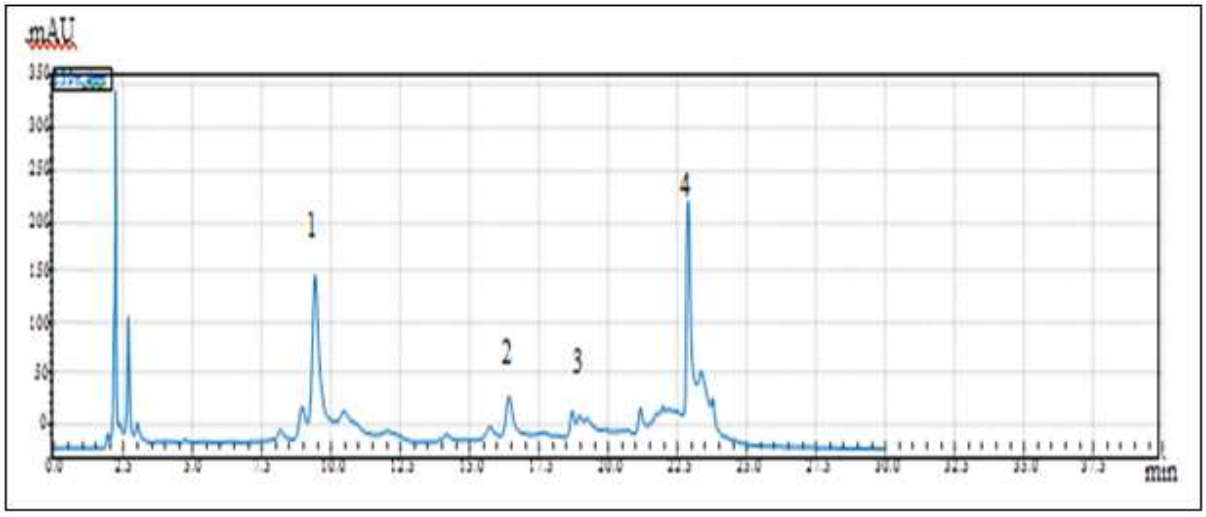

Fig. 1. HPLC chromatogram of a powder fraction (≥100µm µm) of *A. digitata*.

Effect of treatment on creatinine and transaminases (ALAT and ASAT).

The results obtained show firstly that the serum levels of ALAT, ASAT of the negative control rats fed only a hyperlipidic diet are significantly higher ($p<0.05$) than those of the normal control. However, these levels in the groups of animals subjected to the *A. digitata* powder samples were lower than in the negative control.

There was no significant difference in the transaminase levels of the animals subjected to the different powder fractions, the raw powder and the ethanolic extract. These data also show that the creatinine levels of the groups of animals subjected to the different *A. digitata* powder samples are identical to those of the normal control but significantly lower than those of the negative control.

Correlation and PCA

Figure 4 shows the PCA of antihyperlipidemic properties and phenolic content of the powder fractions and ethanolic extract of *A.digitata* pulp. The powder samples, phenolic compound content and resulting antihyperlipidemic properties were projected into a single system. The obtained PCA explains 90.21% of the total variation that exists in this system with a contribution of 72.23% for the F1 axis and 17.98% for the F2 axis. It is observed that the content of phenolic compounds(quercetin, rutin, ferulic acid and acid cafeic) 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, ASAT and ALAT. Indeed, as shown in Fig. 4, the phenolic compounds allowed the increase of serum HDL-C and

the decrease of serum levels of LDL-C, TC, TG, CT/HDL-C and HDL-C/ LDL-C ratios and transaminases in the <50 µm powder fractions and the ethanolic extract. The powder fractions of particle size class 50-100 µm and ≥100 µm as well as the unsieved powder are significantly less rich in phenolic compounds.

Discussion

Phenolic characterization shows that, compared to unsieved powder, the differential separation process improves the concentration of identified compounds. This confirms the hypothesis of Baudelaire (2013) regarding the ability of the successive grinding/sieving process to separate bioactive compounds located in the plant matrix. The LC-MS analyses allowed to observe a differential distribution of phenolic compounds according to particle size with a maximum concentration for the granulometric class <50µm and the ethanolic extract. The particle size ≥100 µm is the only one that systematically leads to a significant decrease in values. In conclusion on these results of phenolic compounds contents, we note firstly that the CDS process favours a higher concentration of phenolic compounds in the finest powder granulometric fraction.

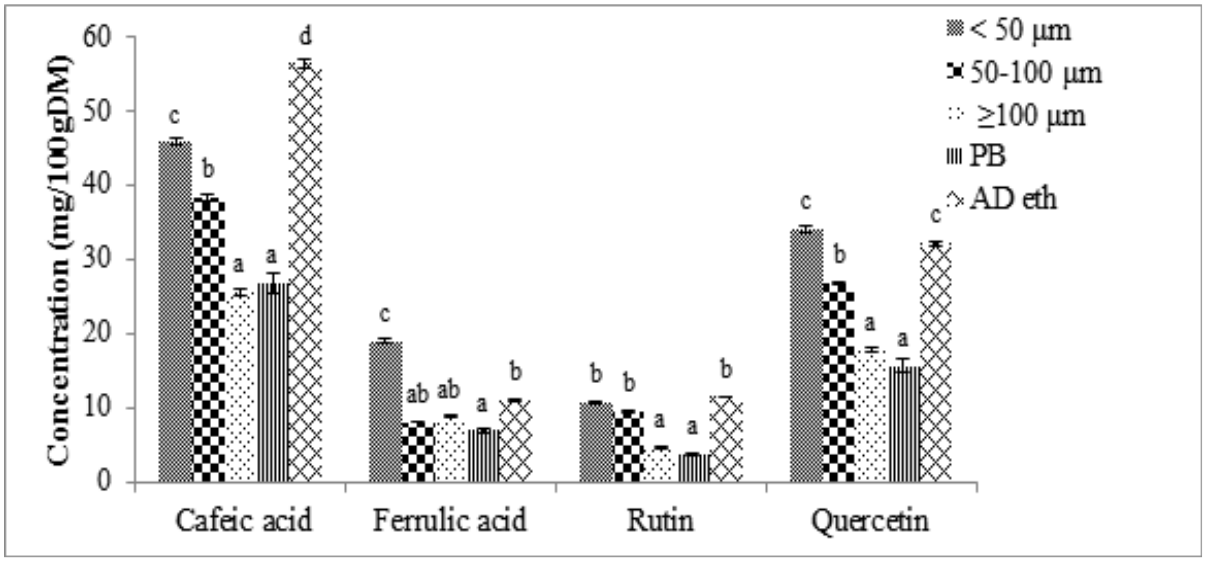

Fig. 2. Contents of phenolic compounds identified in powder fractions obtained by CDS process, unsieved powder and lyophilized ethanolic extract of *A. digitata pulp*.

PB: crude powder; AD eth: ethanolic extract of A. digitata. Histograms with different letters are significantly different at the $p<0.05$ threshold, n=3.

The release of active ingredients from powders seems to be related to the specific surface of the particles. Several reasons can be given to explain this finding, first of all the intracellular localisation of phenolic compounds. Indeed, simple phenolic compounds such as acid are usually covalently bound to polysaccharides of the plant cell wall, forming ester bonds with arabinose groups of the hemicellulose or with a lignin core (Ota *et al.*, 2011). Flavonoids remain in the cytosol where they are synthesised mostly in free form (Kitamura, 2006). To make them bioaccessible, cell wall disruption is necessary. Milling allows the cell to be destructured and the contents of the walls to be dispersed. These results corroborate those of Ngatchic *et al.* (2020) and Zaiter *et al.* (2016), which show that the finest particles concentrate the phenolic compounds best. The physical parameters of the different groups of animals allow us to confirm that the powder fractions and the ethanolic extract of *A. digitata* act effectively on fat reduction, hence the slowing down of weight gain in the tested 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 increase fat excretion. This ability is due to the phenolic compounds which can inhibit pancreatic

lipase activity and lipid absorption, thus contributing to weight management. However, the powder fraction <50 resulted in a greater decrease in weight gain than the other fractions. This can be explained by the fact that the content of phenolic compounds varies from one fraction to another, as we have seen in their quantification. Indeed, the fraction with the highest content of these compounds is the one that allowed a low weight gain. However, this powder fraction does not differ from the ethanolic extract due to the fact that it concentrates the phenolic compounds in an almost identical way.

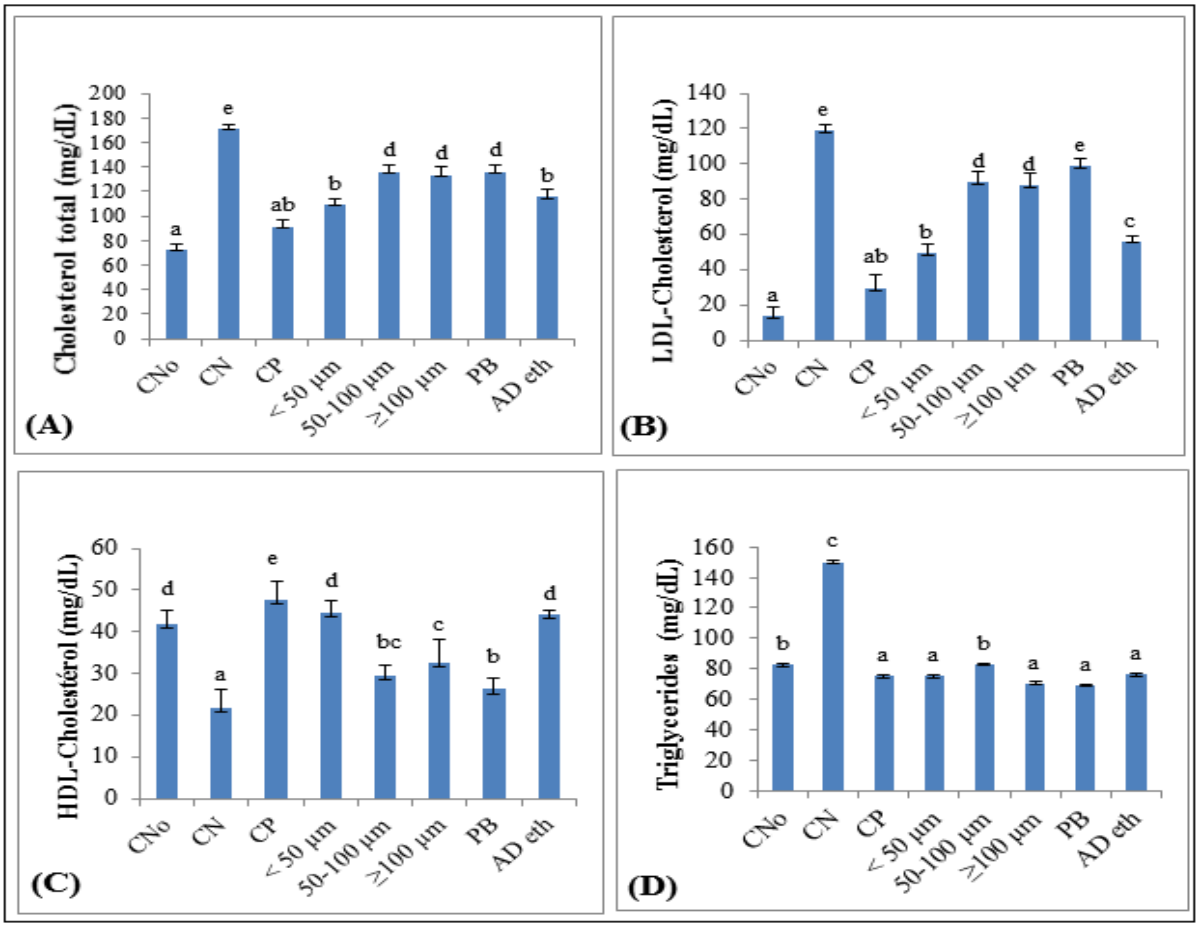

Fig. 3. (A-D): (A) Serum levels of TC, (B) LDL-C, (C) HDL-Cl, (D) TG of rats of *A. digitata* powders (fractions, unsieved powder and lyophilized ethanolic extract) at the end of experimentation**.**

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

In terms of the relative mass of the organs, the results observed confirm the safety of the treatments applied to the plant and the non-toxicity of the dose used in our experiment. This result is in agreement with that of Bouanane *et al.* (2009), which shows that if the values of the organs do not show any difference after a treatment, this would mean that the substance administered is not toxic. The increase in the relative liver mass of the negative control animals is one of the consequences of the excessive consumption of triglycerides which will cause liver dysfunction characterised by an accumulation of fat in the liver (Lewis *et al.*, 2002). This accumulation is at the origin of the increase in liver weight. Finally, the decrease in the relative mass of the testes of the positive control rats is due to the fact that atorvastatin causes various deleterious changes in the histological structure of the testes of adult male albino rats, including a significant decrease in their size (Eman *et al.*, 2008; Sakr *et al.*, 2011).

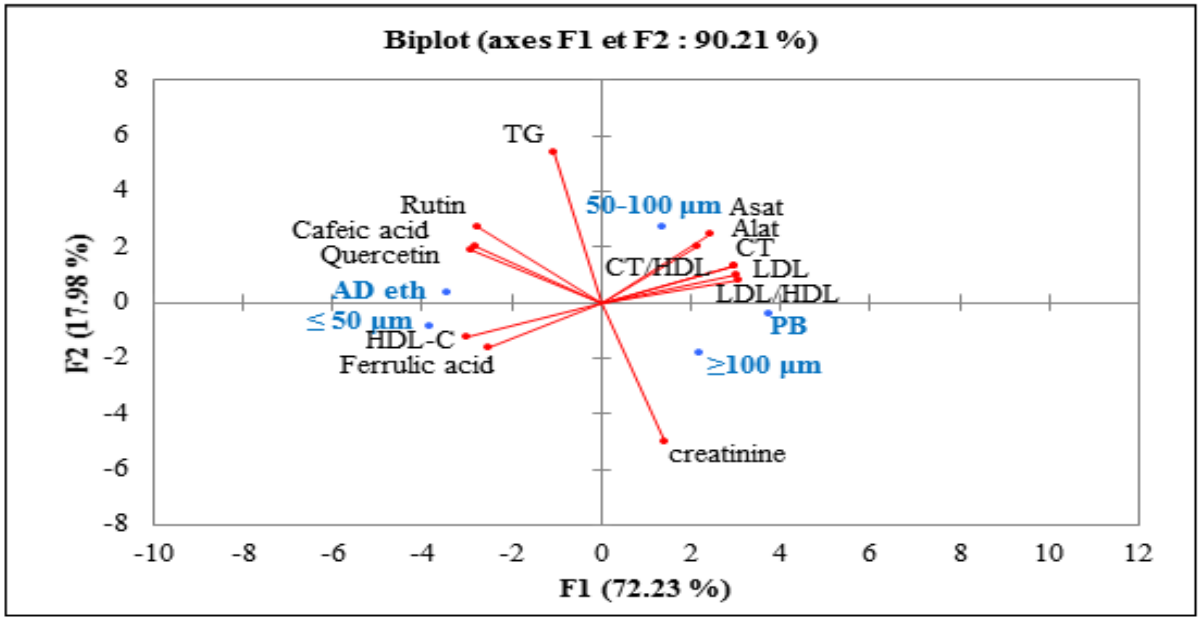

Fig. 4. Principal components Biplot (axes F1 & F2: 90.57 %) analysis of phenolic compound contents and antihyperlipidemic properties of investigated *A. digitata* powders (fractions, unsieved powder, and ethanolic extract*)*.

PB: unsieved powder; AD eth: ethanolic extract.

The reduction in blood levels of TC and LDL-C and TG observed in the groups treated with the powder fractions, crude powder and ethanolic extract of *A. digitata* compared to the negative control is explained by the richness of these plants in phenolic compounds. Numerous studies have shown a correlation between the consumption of these compounds and a decrease in lipid parameters. Among others, we have the work of Nekohashi *et al.* (2014) who demonstrated that quercetin at 5 ml/kg decreased the concentration of cholesterol in rats by inhibiting their absorption. The effect of polyphenol-rich *Lonicera caerulea* berry extract on high cholesterol-induced hypercholesterolemia and changes in lipoprotein metabolites was examined in Caco-2 cells and rats and it was found that Cyanidin-3-glucoside, rutin and caffeic acid which are the main phenolic components of this extract significantly decreased the levels of total Cholesterol, TG and LDL-C but increased the HDL-C level. Quercetin from black tea also inhibits cholesterol absorption by disrupting micelle formation (Ikeda *et al.*, 2010). Indeed, these molecules may act by catalyzing the conversion of HMG-CoA to mevalonate which is an early step limiting cholesterol synthesis. Decreased cholesterol synthesis stimulates the expression of the LDL receptor gene by lifting the negative feedback from intracellular cholesterol. Thus, the increase in LDL receptor synthesis leads to an increase in plasma LDL uptake and catabolism by cells, and thus a decrease in circulating LDL levels (Laviolle *et al.*, 2012). These compounds also have the ability to decrease lipid absorption by inhibiting pancreatic lipase, which is the most important enzyme for efficient triglyceride digestion (Ngatchic *et al.*, 2020; SHI *et al.*, 2014). They can act directly on the site of the active enzyme or, indirectly, by increasing the size of the lipid (TG) droplets, thus reducing the accessibility of the substrate to the enzyme. This effect may limit the intestinal absorption of triglycerides. The serum levels of lipid parameters vary from one particle size class to another due to the fact that the distribution of phenolic compounds varies from one particle size class to another with a better activity for fraction 50. However, compared to ethanolic extract this fraction acts in the same way as ethanolic extract on the lipid parameters measured with the exception of LDL-cholesterol levels. This suggests that CDS process has improved the protective effect of *A. digitata* against

hyperlipidaemia as much as ethanol extraction. Creatinine represents the major biological marker of renal impairment (Jodynis-Liebert *et al.*, 2010). 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 impairment of glomerular filtration and renal dysfunction, a factor leading to CVD (Mukinda *et al.*, 2010). While ASAT and ALAT are important markers of liver function, an organ that is essential in maintaining cholesterol homeostasis.

The assessment of these two parameters helps to determine possible abnormalities caused by hyperlipidemia (Sookoian and Pirola, 2015). ALAT is a liver-specific enzyme, making it an important and sensitive indicator of hepatotoxicity. ASAT is also an indicator of hepatocyte destruction although in addition to the liver, it is found in the heart, skeletal muscle, lung and kidney (Dufour *et al*, 2000). Serum levels of ALAT and ASAT rise rapidly when the liver is damaged (Pratt and Kaplan 2000). The results show that the hepatoprotective and nephroprotective effects as they protected the liver and kidneys from hyperlipidic diet-related damage as much as the ethanolic extract.

Conclusion

The effect of the controlled differential sieving process on the phenolic compounds and antihyperlipidemic activity of *A. digitata* pulp was studied and compared with that of the ethanolic extract in this work. This process allowed a better concentration of these compounds and consequently a better activity in the obtained powders and particularly for the fraction <50 µm compared to the unsieved powder. The content of phenolic compounds and the antihyperipidemic activity of this fraction are almost similar to that of the ethanolic extract. With the exception of ferrulic acid which is more concentrated in the fraction<50 µm and caffeic acid which is more concentrated in the ethanolic extract. The controlled differential sieving process can be an alternative to ethanol extraction in the concentration of phenolic compounds and allow a good management of CDV.

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Conflict of interest

The authors declare no conflict of interest.

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