Lowering cholesterol bioaccessibility and serum concentrations by saponins: *in vitro* and *in vivo* studies

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Supplementary information

I. Methods.

S1. Extraction of the non-hydrolyzed TG and reaction products by chloroform.

The used procedure is taken from ref. (26). Briefly, after stopping the lipolysis reaction with Orlistat granules, the reaction mixture was allowed to cool down to room temperature and its pH was decreased to pH = 2 by adding HCl (to decrease the solubility of the reaction products in the aqueous phase). Next, 6 mL chloroform was added and the sample was sonicated for 15 min. After every 5 min of sonication, the sample was agitated by vigorous hand shaking. The obtained complex dispersion was centrifuged for 30 min at 3620 g (4500 rpm), which led to separation of clear aqueous and organic phases, indicating that the oily drops were entirely extracted by the chloroform phase. The recovery of cholesterol, FA, MG, DG and TG by this extraction procedure was ≥ 90 %.

The same extraction procedure was applied to the aqueous phases, separated by filtration or centrifugation. The obtained chloroform phase was further analyzed by GC.

S2. Gas chromatography (GC) procedure.

The GC analyses were performed on a TRACE GC apparatus (ThermoQuest, Italy) equipped with autosampler AS 2000. We used a capillary column Quadrex, USA, with the following specification: 5 % phenyl methylpolysiloxane, 10 m length, I.D. 0.53 mm, 0.1 μm film thickness. Cold on-column injection was used, at a secondary cooling time of 0.3 min. The injection volume was 1 μL. The oven was programmed as follows: start at 120 ºC, hold 2 min, ramp 1 to 325 ºC at 10 ºC/min, ramp 2 to 345 ºC at 5 ºC/min, hold 5 min. The flame-ionization detector (FID) temperature was set to 350 ºC. The carrier gas was helium, set at a constant pressure flow mode (60 kPa).

Illustrative chromatogram, obtained in real experiment by the procedure described above, is presented in Figure S1. The different peaks were identified using the standard substances. Hexadecanol (cetanol) was used as internal standard, at a concentration of 0.15 mg/mL.

S3. Procedure for quantitative analysis of the GC data. The chromatograms obtained by the GC analysis, described in the previous Section, were analysed using the peak areas. The concentration of the different FA, MG, TG and cholesterol was calculated using internal standard, by the following equation:

$$C_X = A_X \frac{C_{IS}}{A_{IS}} \tag{S1}$$

where C_X is the component concentration, A_X is the component peak area, C_{IS} is the concentration of the internal standard, and A_{IS} is the peak area of the internal standard. The experiments performed with standard solutions, containing known quantities of FA, MG, TG and cholesterol, showed that correction factors of 1.16 and 1.90 are needed for the FA and the TG, respectively. No correction factor was needed for the MG and no DG was found in any of the samples studied.

S4. Atomic absorption spectrometry (AAS) for determination of Ca^{2+} ions in Quillaja Dry and Sapindin.

QD or SAP extract was dissolved in an electrolyte solution containing 1.37 M NaCl, 0.1 M KCl, 25 mM Na_2EDTA and 2 g/L NaN_3, because Na^+ and K^+ ions interfere with calcium measurements by AAS (see J. B. Willis, The determination of metals in blood serum by atomic absorption spectroscopy: calcium, *Spectrochim. Acta*, 1960, 16, 259–272). A Perkin Elmer Analyst 400 flame atomic absorption spectrometer was used for determination of the calcium concentration in the obtained solution. A calcium hollow cathode lamp was used as a specific radiation source. The sample was measured directly in air/acetylene flame, under optimal instrumental parameters.

Standard solutions of calclium(II) ions were prepared by dissolving known amounts of calcium dichloride in an electrolyte solution, which had the

same composition as the one used for QD and SAP. In this way, we prepared matrix matched standards. A calibration curve was prepared by measuring the absorption of the standard solutions. The obtained dependence of the absorbance on the Ca^{2+} concentration was linear and was used to calculate the calcium in QD and SAP extracts.

S5. Degree of TG hydrolysis. The digestion of edible oils or fats by the pancreatic lipase occurs via consecutive reactions, where the TG is hydrolyzed to DG and MG (Verger, R. 1984. Pancreatic lipase. In Lipases. B. Borgström and H. L. Brockman, editors. Elsevier, Amsterdam, 84–150). To quantify the extent of TG lipolysis at the end of the experiment, we use the degree of total TG lipolysis, α, defined as:

$$\alpha = \frac{C_{TG}^{INI} - C_{TG}}{C_{TG}^{INI}} \tag{S2}$$

Here C_{TG}^{INI} is the initial molar concentration of TG (which is known in advance), and C_{TG} is the molar concentration of the remained, non-hydrolyzed TG, as determined by GC. The value of α accounts for the relative amount of TG that has been transformed into both MG and DG.

The degree of TG transformation to DG is defined as:

$$\beta = C_{DG} \big/ C_{TG}^{INI} \tag{S3}$$

Here C_{DG} is the molar concentration of the formed and non-hydrolyzed DG. The value of β accounts for the amount of TG, which is transformed into DG, without further transformation to MG.

The degree of TG transformation to MG is defined as:

$$\gamma = C_{MG} \big/ C_{TG}^{INI} \tag{S4}$$

In practice, we determined the values of α from the initial and the final TG concentrations, eq. (S2), and the values of β and γ were determined from C_{MG} and C_{DG}, using eqs. (S3) and (S4), respectively.

6S. High pressure liquid chromatography (HPLC) for analysis of bile salts solubility. The analysis was performed on a Shimadzu instrument (Japan) equipped with two high-pressure mixing binary gradient pumps (LC-20AD), DGU-14A four-line membrane degasser, SIL-10ADvp autosampler, CTO-10ASvp wide temperature range column oven and SPD-10Avp UV-VIS detector. The separation was carried out on 25cm × 4.6 mm i.d., 5 μm particle size, Ascentis® C18 column (Supelco, USA) and a guard column Ascentis® C18 Supelguard™ Guard Cartridge (5 μm particle size, 2 cm × 4 mm i.d.). All samples were filtered through 200 nm filters (section 1.2.4) and kept at 37 ºC prior injection.

For the separation of bile acids we modified a procedure from Supelco technical application G005679. We used gradient elution with 1.5 vol. % formic acid in water (A) and 1.5 vol. % formic acid in acetonitrile (B). The total flow rate was kept at 1 mL/min during the entire period of the analysis (35 min). The system was run with the following gradient program: from 40 % B to 75 % B for 20 min, from 75 % B to 40 % B for the next 2 min and constant flow at 40 % B for 13 min. The sample injection volume was 20 μL. The UV-VIS detector was set at λ = 200 nm and the column temperature was 30 ºC.

Table S1. Fatty acid composition of the studied fats and oils, as determined by GC analysis (section 2.6). These percentages are defined as the moles of given fatty acid, normalized by the total moles of all visible fatty acids in the GC chromatogram. The fatty acid composition, obtained after complete hydrolysis to glycerol with alcoholic NaOH, is abbreviated "To Gly". Our results are compared to literature data obtained from AOCS (Food fats and oils 9th edition, Institute of shortening and edible oils, 2006, p. 354). The composition obtained after *in vitro* lipolysis is abbreviated "After Lip". The difference between the results "To Gly" and "After Lip" is due primarily to the fact that the pancreatic lipase hydrolyzes FA located in 1 and 3 positions of the TG.

Fatty acids, molar %	**SFO**			**Cocoa butter**		
	To Gly	**To Gly (Lit.)**	**After Lip**	**To Gly**	**To Gly (Lit.)**	**After Lip**
Palmitic acid (C16:0)	8	7	11	29	26	37.5
Oleic + linoleic acid (C18:1,2)	87	88	82	34	37	15
Stearic acid (C18:0)	5	5	7	37	35	47.5
Saturated FA (SFA)	13	12	18	66	61	85
Unsaturated FA (UFA)	87	88	82	34	37	15

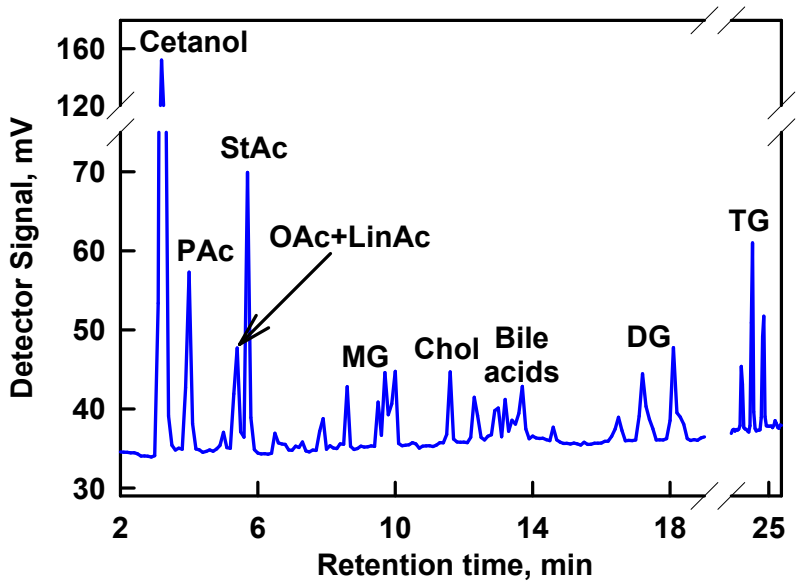

Figure S1. GC detector signal, as a function of retention time, for a cocoa butter emulsion, after *in vitro* lipolysis experiments and extraction with chloroform. The different peaks were assigned using the respective substances as standards.

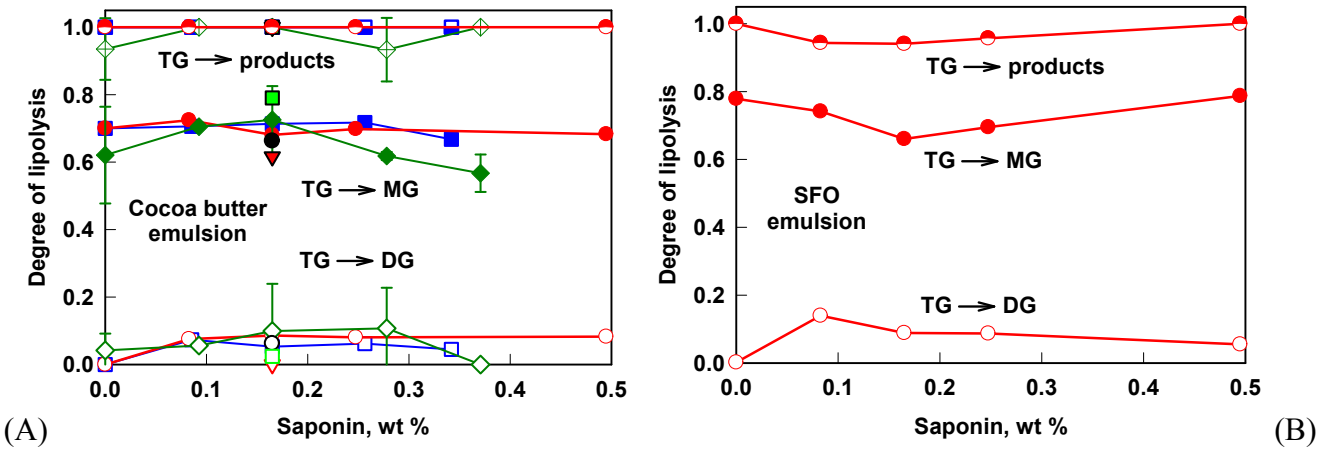

Figure S2. Degrees of TG lipolysis, as a function of weight concentration of saponin, for experiments performed with (A) Cocoa butter emulsion and (B) SFO emulsion, in presence of QD (red circles), SAP (blue squares), ASC (green diamonds), GS (triangle down), FEN (green squares) and ESC (black circles). Overall degree of TG lipolysis (semi filled symbols), degree of lipolysis to monoglycerides (full symbols) and degree of lipolysis to diglycerides (empty symbols).

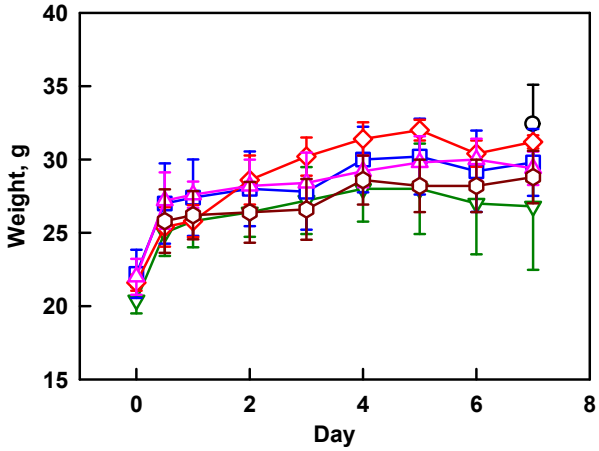

Figure S3. Average mouse weight in each group, versus day of experiment for control group, fed with standard rodent food (circles) and groups fed with food, supplemented with: cocoa butter (green triangles down), cocoa butter + cholesterol (red diamonds), cocoa butter + cholesterol + QD (brown hexagons), cocoa butter + SAP (blue squares), cocoa butter + cholesterol + SAP (pink triangles up).

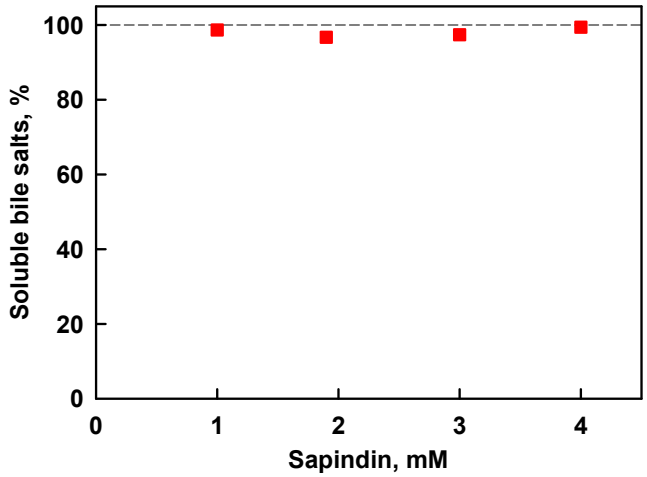

Figure S4 Percentage of soluble bile acids, as a function of molar Sapindin concentration, as measured *in vitro*. The horizontal dashed line corresponds to the concentration of bile salts in the absence of saponins.