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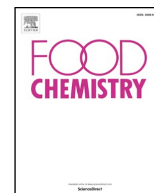
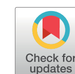


NMR-based *Lavado* cocoa chemical characterization and comparison with fermented cocoa varieties: Insights on cocoa's anti-amyloidogenic activity



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ABSTRACT

The metabolic profile of *Lavado* cocoa was characterized for the first time by NMR spectroscopy, then compared with the profiles of fermented and processed varieties, *Natural* and commercial cocoa.

The significant difference in the contents of theobromine and flavanols prompted us to examine the cocoa varieties to seek correlations between these metabolite concentrations and the anti-amyloidogenic activity reported for cocoa in the literature.

We combined NMR spectroscopy, preparative reversed-phase (RP) chromatography, atomic force microscopy, *in vitro* biochemical and cell assays, to investigate and compare the anti-amyloidogenic properties of extracts and fractions enriched in different metabolite classes.

Lavado variety was the most active and the catechins and theobromine were the chemical components of cocoa hindering Aβ peptide on-pathway aggregation and toxicity in a human neuroblastoma SH-SY5Y cell line.

1. Introduction

The significant consumption of cocoa worldwide and the evidence of a number of beneficial effects of cocoa for human health (Magrone, Russo, & Jirillo, 2017), including antioxidant, anti-inflammatory and neuroprotective activities (Cimini et al., 2013; Nehlig, 2013) prompted us to investigate several cocoa varieties.

Cocoa, the dried seed of *Theobroma cacao*, is a rich source of polyphenols (12–18% dry weight); among these flavon-3-ols (or flavanols) are well-known brain-bioavailable, bioactive polyphenols (Dubner, Wang, Ho, Ward, & Pasinetti, 2015). The seed contains mainly the monomeric flavan-3-ols catechin and epicatechin, but also more complex ones such as the polymeric procyanidins (Neilson & Ferruzzi, 2011).

During its industrial transformation, cocoa can be processed by different methods and the processing can dramatically affect the polyphenol and flavanol contents, which may ultimately influence bioactivity (Hurst et al., 2011). *Natural* cocoa is the powder obtained after fermenting, drying and roasting cocoa beans. Then the Dutch processing can be employed, treating cocoa powder with alkali and heat, to change the color and flavor of cocoa products. Fermentation and Dutch processing – the basis of most of the common chocolate production processes – result in the loss of up to 90% of the cocoa flavanols. However, *Lavado* cocoa, the unfermented cocoa immediately hand-washed and sun-dried after harvesting, contains the largest number of polyphenols and should therefore be the most interesting product from a nutraceutical point of view (Hurst et al., 2011).

Nevertheless, to the best of our knowledge, *Lavado* cocoa has not yet been thoroughly investigated by NMR. NMR spectroscopy is a suitable technique for identifying and characterizing the main metabolites in cocoa. NMR has been used to characterize the metabolic profile of cocoa extracts and to compare cocoa of different origins and/or processed with different levels of fermentation (Caligiani, Acquotti, Cirlini, & Palla, 2010; Caligiani, Palla, Acquotti, Marseglia, & Palla, 2014; Marseglia et al., 2016). However, all these analyses only examined fermented cocoa beans. We therefore used NMR spectroscopy to obtain the metabolic profiles of *Lavado* cocoa extracts after different extraction procedures. We also compared this variety to *Natural* and commercial ones, highlighting its significant content in flavanols.

As already reported by our group (Sironi et al., 2014), catechins in

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green tea extracts can bind and inhibit the aggregation of Aβ peptides. Cocoa extracts have also been reported to inhibit the oligomerization of Aβ peptide (Wang et al., 2014) and Aβ oligomer-induced cytotoxicity in PC12 cells (Heo & Lee, 2005). Nevertheless, the extract components responsible for this specific biological activity have never been identified. In this context, the identification in cocoa extracts of ligands and inhibitors of Aβ oligomers can be essential to confirm cocoa potential neuroprotective effect and to dissect the mechanisms of action at the molecular level.

To identify food and beverages containing potential anti-amyloidogenic compounds, an experimental protocol based on NMR spectroscopy combined with other biophysical and biochemical assays, was developed to detect ligands of amyloid aggregates in natural edible matrices. This approach was effective in identifying amyloid aggregate ligands in sage (Airoldi et al., 2013), green tea (Sironi et al., 2014), coffee (Ciaramelli et al., 2018) and *Radix imperatoria* (Palmioli et al., 2019). Here we applied the same experimental approach to screen cocoa extracts for the presence of ligands and modulators of Aβ peptides, particularly the soluble and cytotoxic Aβ1-42 oligomers involved in AD etiology (Haass & Selkoe, 2007).

We examined the anti-amyloidogenic activity of cocoa extracts in biochemical and cellular assays. In addition, we employed STD-NMR to investigate the interactions of the extracts and their enriched fractions with Aβ1-42 oligomers: catechins and theobromine in cocoa bound Aβ oligomers and inhibited their further aggregation, thus preventing their neurotoxicity.

2. Material and methods

2.1. Preparation of cocoa extracts

Natural cocoa beans from Ghana and *Lavado* cocoa beans from Mexico (nibs only or whole beans: nibs and shells) were finely ground under liquid nitrogen and the powder was extracted. Industrially processed cocoa ("cacao amaro" from a local Carrefour supermarket, Boulogne-Billancourt, France) was bought as a powder so it did not require grinding. All reagents and solvents were reagent grade, from Sigma-Aldrich unless indicated otherwise (Sigma-Aldrich, St. Louis, MO, USA). A defatting step was run before the extraction in some cases. Briefly, the ground cocoa powder (5 g) was extracted in a Soxhlet apparatus (15 cycles) using dichloromethane (250 mL) to remove the lipid fraction. The solid was dried and used for subsequent extractions.

Different extraction procedures were employed to obtain cocoa extracts. Extractions were repeated three times for each cocoa sample.

Extraction in boiling water (Heo & Lee, 2005). Cocoa powder (1 g) was suspended in 5 mL of MilliQ water under magnetic stirring, at 100 °C for 2 min. The cocoa extracts were centrifuged at 15,000 × *g* (ScanSpeed 1730R Labogene, Lynge, Sweden) for 5 min at 4 °C and the supernatants were filtered through 0.45 μm PTFE filters (Pall Corporation, Port Washington, NY, USA), lyophilized (Christ ALPHA 1-2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and weighed. Dry extracts were stored at −20 °C until use.

Extraction with water using a Soxhlet apparatus (Arlorio et al., 2008). The defatted cocoa powder (5 g) was extracted for 10 h in an automatic Soxhlet apparatus, with MilliQ water at boiling point as the solvent (250 mL). The extract was lyophilized (Christ ALPHA 1–2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), weighed and stored at −20 °C.

Extraction with methanol using a Soxhlet apparatus. The defatted cocoa powder (5 g) was extracted for 10 h in an automatic Soxhlet apparatus, with methanol as the solvent (250 mL). The solvent was evaporated to dryness (under *vacuum* at 40 °C), the solid was suspended in water, lyophilized (Christ ALPHA 1–2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and weighed. Dry extracts were stored at −20 °C until use.

Hydro-alcoholic extraction with boiling water/methanol (8:2 v/v) (Caligiani et al., 2010, 2014): 200 mg of cocoa powder (defatted or not defatted) were extracted with 20 mL of a MilliQ water/methanol mixture (8:2 v/v), kept at boiling point for 10 min in an oil bath with a reflux apparatus under magnetic stirring. Extracts were cooled, filtered on a Buchner apparatus under vacuum (Whatman filter paper grade 4, por. 0.20–0.25 μm), then through 0.45 μm PTFE filters (Pall Corporation, Port Washington, NY, USA). Methanol was evaporated (under vacuum at 40 °C) and the water portion was lyophilized (Christ ALPHA 1–2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and weighed. Dry extracts were stored at −20 °C until use. Extraction was repeated as described also on 500 mg and 1 g of powder with respectively 50 mL and 100 mL of extraction mixture.

Hydro-alcoholic extraction with water (pH 4.5)/methanol 7:3 (Amigoni et al., 2017): 400 mg of ground cocoa sample were extracted with 40 mL of a mixture of acidified (with 1 M HCl) MilliQ water (pH 4.5, 70%) and methanol (30%) by sonication at 37 kHz for 15 min in an ultrasound bath (Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) at 30 °C. The solution was filtered through cotton wool and 0.45 μm PTFE filters (Pall Corporation, Port Washington, NY, USA), concentrated under reduced pressure at 40 °C and freeze-dried (Christ ALPHA 1-2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The lyophilized sample was weighed and stored at −20 °C.

Extraction with acetone/water/acetic acid (70:29.5:0.5). Cocoa powder (500 mg) was extracted with 20 mL of a mixture of acetone/water/acetic acid (70:29.5:0.5) by sonication for 10 min at 37 kHz in an ultrasound bath (Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany), at 35 to 50 °C. It was filtered through cotton wool and 0.45 μm PTFE filters (Pall Corporation, Port Washington, NY, USA) and evaporated under vacuum. It was then re-dissolved in 20 mL of water and freeze-dried (Christ ALPHA 1-2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), weighed and stored at −20 °C until use.

2.2. Ultrafiltration

The freeze-dried sample of the hydro-alcoholic cocoa extract was dissolved in MilliQ water/MeOH 8:2 (10 mg/mL) and centrifuged (14,500 × *g*, 10 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). The supernatant was ultrafiltered in tangential flow ultrafiltration devices with a polyethersulfone membrane and molecular weight cut-off (MWCO) 3 kDa (Vivaspin 500, GE Healthcare, Little Chalfont, Buckinghamshire, UK) through a centrifuge (14,500 × *g*, 30 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). Ten ultrafiltration steps were run on 1 mL of sample, collecting the ultrafiltrate low-molecular-weight (LMW) fraction. MeOH was evaporated from the LMW fraction (MW < 3 kDa) under vacuum and the sample in water was freeze-dried (Christ ALPHA 1-2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), weighed and stored at −20 °C.

The same procedure was applied to cocoa samples obtained with Soxhlet extraction in water, after dissolution in MilliQ water at 10 mg/mL.

2.3. Preparative reverse phase C18 column chromatography

Automated flash chromatography was done on a Biotage® Isolera™ Prime system (Biotage AB, Uppsala, Sweden) equipped with a Spektra package. A solution of the extracted sample (200 mg in 5 mL of MilliQ water) was loaded into a SNAP KP-C18-HS (12 g) cartridge, equipped with a pre-column Biotage's Samplet® cartridge SNAP-C18 (1 g). Column chromatography was run with water (solvent A) and methanol (solvent B) as eluent solvents. A linear elution gradient was applied (2% B for 2 CV, 2% to 100% of B in 15 CV and 100% B for 3 CV) at a flow rate of 12 mL/min. The eluate was automatically collected in fractions

based on photodiode array detector signal (range 200–400 nm) and UV detection at λ = 280 nm and λ = 320 nm. Fractions were pooled in homogenous groups; organic solvent was removed under reduced pressure and residues were freeze-dried (Christ ALPHA 1-2 LD PLUS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) obtaining fractions A-E.

2.4. NMR spectroscopy

Freeze-dried samples were suspended in deuterated solvent (D_2O or MeOD or D_2O/MeOD 8:2, EURISO-TOP, Saint-Aubin Cedex, France) at a final concentration of 5 to 15 mg/mL. All samples were sonicated (37 kHz, 10 min, Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) and centrifuged (15000 *xg*, 10 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). 4,4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, final concentration 1 mM) was added to the supernatant as an internal reference for concentrations and chemical shift. The pH of each sample was verified with a microelectrode (InLab Micro, Mettler Toledo, Columbus, OH, USA) and adjusted to 7.0 with NaOD or DCl. All pH values were corrected for the isotope effect. The acquisition temperature was 25 °C. All spectra were acquired on an AVANCE III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H lock) cryogenic probe. ^{1}H NMR spectra were recorded with *noesygppr1d*, *cpmgpr1d* and *ledbpgppr2s1d* pulse sequences in Bruker library and 256 scans, spectral width 20 ppm, relaxation delay 5 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. Chemical shifts were internally calibrated to the DSS peak at 0.00 ppm. The ^{1}H,^{1}H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 48 scans and 512 increments, 80 ms mixing time and relaxation delay 2 s. ^{1}H,^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 512 increments, relaxation delay 2 s.

NMR spectra processing and peak peaking were done using the MNova software package of Mestrelab (MestReNova v 12.0.4-22023, 2018, Mestrelab Research, Santiago de Compostela, Spain). For metabolite quantification, the SMA analysis tool integrated in the MestreNova software package (MestReNova v 12.0.4-22023, 2018, Mestrelab Research, Santiago de Compostela, Spain) was employed to set a semi-automatic protocol for metabolite identification and quantification, building specific libraries for the matrices analysed. This protocol employed the GSD (global spectrum deconvolution) algorithm: overlapping regions were deconvoluted and absolute quantification was possible for metabolites with resonances in crowded spectral areas too. When feasible, the compound concentration was calculated from the mean of the different assigned signals.

2.5. Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated as mean of the total polyphenols and radical scavenging ability and measured by three spectrophotometric methods, as previously reported (Amigoni et al., 2017; Ciaramelli, Palmioli, & Airoldi, 2019; Palmioli et al., 2019). Preliminarily the UV–Vis absorbance profile was determined. Each extract was dissolved at 50 μg/mL in MilliQ water and the measurement was recorded at room temperature. Absorbance was measured with a Varian Cary® 50 Scan UV–Visible Spectrophotometer (Agilent, Santa Clara, CA, USA) using disposable polymethyl methacrylate (PMMA) or quartz semi-micro 10 mm cuvettes, relative to a blank solution. Data were reported as means (± SD) of triplicate measurements in two independent evaluations. The total polyphenol content was determined with the Folin Ciocalteu assay, as previously reported (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, 80 μL of diluted samples (or standards/blank) and 40 μL of Folin's reagent (Sigma-Aldrich, St. Louis, MO, USA) were dispensed in a cuvette containing 400 μL of MilliQ water. Then 480 μL of Na_2CO_3 10.75% (w/v) solution was added and after 30 min of incubation at room temperature absorbance was read at 760 nm. Samples were diluted to 0.5 mg/mL and standard solutions (0–200 μg/mL) of Gallic acid were used for calibration (linear fitting $R^2 > 0.99$, n = 7). Results were expressed as mg of Gallic acid Equivalent (GAE eq) per g of freeze-dried cocoa extract.

The radical scavenging ability of the extract was determined by ABTS-TEAC and DPPH assay. The ABTS-TEAC assay is based on the evaluation of the scavenging capacity of an antioxidant to the long-life colored cation $ABTS^{\cdot+}$ (Re et al., 1999). Briefly, a 7 mM stock solution of $ABTS^{\cdot+}$ was produced by mixing equal amounts of a 14 mM ABTS solution and a 4.9 mM $K_2S_2O_8$ solution in MilliQ water (final concentrations 7.00 mM and 2.45 mM, respectively). The mixture was left at room temperature in the dark for at least 12–16 h before use and stored at 4 °C for 7 days.

A working solution of $ABTS^{\cdot+}$ was prepared daily by diluting the stock solution (1:50), reaching 0.70 ± 0.05 absorbance at 734 nm. Fifty μL of the sample (or standards) were added in a cuvette containing 950 μL of $ABTS^{\cdot+}$ solution, and the absorbance at 734 nm was read after 30 min of incubation at room temperature. Samples were diluted to 0.25 mg/mL and standard solutions (0–500 μM) of Trolox were used for calibration (linear fitting $R^2 > 0.99$, n = 7). Results were expressed as mmol of Trolox equivalent (TE) *per* g of freeze-dried cocoa extract.

The DPPH assay is based on the scavenging of the stable free-radical 2,2-diphenyl-1-picrylhydrazyl, according to the literature. (Sharma & Bhat, 2009). Briefly, 950 μL of a diluted solution of DPPH in buffered MeOH (100 μM in a mixture of 60% MeOH and 40% acetate buffer, pH 4.5, Abs 0.70 ± 0.05) and 50 μL of a diluted sample (or standard) were placed in a cuvette, and the absorbance at 517 nm was read after 30 min of incubation at room temperature. Samples were diluted to 0.25 mg/mL and standard solutions (0–500 μM) of Trolox were used for calibration (linear fitting $R^2 > 0.99$, n = 7). Results were expressed as mmol of Trolox equivalent (TE) per g of freeze-dried cocoa extract.

2.6. Peptide synthesis

Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV-VIA) was prepared by solid-phase peptide synthesis (SPPS) on a 433A Syro I synthesizer (Applied Biosystems, Foster City, CA, USA) using Fmoc-protected L-amino acid derivatives, NovaSyn-TGA resin (Novabiochem, Sigma-Aldrich, St. Louis, MO, USA) and a 0.1 mM scale. The peptide was cleaved from the resin as previously described (Manzoni et al., 2009), and purified by reverse phase HPLC on a semi-preparative C4 column (Waters, Milford, MA, USA) using water: acetonitrile gradient elution. Peptide identity was confirmed by MALDI-TOF analysis (model Reflex III, Bruker, Billerica, MA, USA). The purity of peptides was always above 95%.

2.7. Thioflavin t binding assay

Aβ1-42 were dissolved in 10 mM NaOH, H_2O and 50 mM PB (1:1:2) to 2.5 μM with or without the cocoa extracts (15.6 μg/ml) and the L1 cocoa fractions A, B, C and D (12.5 μg/ml), and were incubated at 37 °C in 20 μM ThT (Sigma-Aldrich, St. Louis, MO, USA) in 96-well black plates (Isoplate, Perkin Elmer, Waltham, MA, USA). The ThT fluorescence was monitored for 24 h with a plate reader (Infinite F500 Tecan: excitation 448 nm, emission 485 nm, 37 °C). Data were expressed as the mean of three replicates, calculated by subtracting the relative control solutions (fractions alone) and were expressed as the percentage reduction of Aβ1-42 aggregation.

2.8. Atomic force microscopy (AFM)

Aβ1-42 was dissolved as previously described to 2.5 μM with or without the cocoa fraction (12.5 μg/mL) and incubated in quiescent conditions at 37 °C for 24 h. After the incubation, 30 μL of samples were spotted onto a freshly cleaved Muscovite mica disk and incubated for 7 min. The excess sample on the disk was washed with 10 mL MilliQ

water and dried under a gentle nitrogen stream. Samples were mounted onto a Multimode AFM with a NanoScope V system (Veeco/Digital Instruments, Plainview, NY, USA) operating in Tapping Mode and measurements were made using 0.01–0.025 Ohm/cm antimony-doped silicon probes (T: 3.5–4.5 μm, L: 115–135 μm, W: 30–40 μm, k: 20–80 N/m, f0: 323–380 kHz, Bruker AFM probes) with a scan rate in the 0.5–1.2 Hz range, proportional to the area scanned.

Measurements confirmed all the topographic patterns in at least four separate areas and to exclude interference from any artefacts freshly cleaved mica DISCS soaked with 30 μL of PB 50 mM were also analyzed as controls. Samples were analyzed with the Scanning Probe Image Processor (SPIP Version 5.1.6 released April 13, 2011) data analysis package.

2.9. Cell cultures

The human neuroblastoma SH-SY5Y cell line was grown in Dulbecco's Modified Eagle's medium-F12 (DMEM, Lonza, Basel, Switzerland) supplemented with L-glutamine (5 mM, Gibco, Invitrogen, Waltham, MA, USA), antibiotics (penicillin/streptomycin 10,000 U, Lonza, Basel, Switzerland) and 10% heat-inactivated fetal calf serum (FCS, Gibco, Invitrogen, Waltham, MA, USA), at 37 °C in 5% CO_2 in the air.

2.10. In vitro toxicity assay

Cytotoxicity was evaluated after 24 h incubation using the MTT reduction assay. SH-SY5Y cells were seeded in 96-well plates (10^5 cell/mL) and incubated overnight (37 °C, in a humidified 5% CO_2 atmosphere) in 1% FCS-containing medium to reduce cell growth. Aβ1-42 was dissolved in 10 mM NaOH, H_2O and PBS (1:1:2) and added to the cocoa extracts (62.5 μg/mL) and the L1 cocoa fractions A, B, C and D (50 μg/mL) before treatment of the SH-SY5Y cells, to the final concentration of 10 μM for Aβ1-42 in the well. Tetrazolium (20 μL of 5 mg/mL, Sigma Aldrich) was added to each well and incubated for 4 h. The medium was replaced with acidified isopropanol (0.04 M HCl) to dissolve the purple precipitate and the absorbance intensity was measured at 570 nm, using a plate reader (Infinite M200, Tecan, Männedorf, Switzerland). Data were expressed as percentages of controls (solvent), with three separate replicates.

2.11. Assessment of autophagy markers

After 24 h exposure to cocoa extracts (L1 extract and fraction D, 50 μg/mL) SH-SY5Y cells were pelleted and lysed in cell extraction buffer (Invitrogen, Waltham, MA, USA) supplemented with 1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA), protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and the protein concentration was determined by Bradford's method. Sample lysates were diluted in Laemmli's loading buffer pH 6.8, denatured at 95 °C for 4 min, separated by SDS-PAGE in 4–12% Tris-glycine gels (Invitrogen, Waltham, MA, USA) or 15% home-made gels, and transferred to nitrocellulose. Blots were blocked for 1 h, incubated overnight at 4 °C with specific primary antibodies: anti-Beclin-1 (Cell Signaling, dilution 1:1000) or anti-LC3B (Cell Signaling, dilution 1:500) or anti-LAMP2A (Abcam, dilution 1:900) or anti-HSC70 (Abcam, dilution 1:3000), then with HRP-linked anti-mouse or -rabbit IgG antibody (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. An anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA; dilution 1:40000) was used as internal standard.

Signals were revealed by chemiluminescence, detected using the ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA, USA) imaging system and quantified using ImageJ software. Protein expression was calculated as the ratios between the optical densities of the target protein and internal standard and expressed as percentages of the mean of the vehicle group.

2.12. Statistical analysis

All data are shown as mean ± standard deviation (SD). Repeated measures ANOVA, followed by Dunnett's multiple comparison test, was used to assess the significance of differences among groups. Statistical analysis was done using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA).

2.13. NMR spectroscopy binding

To obtain samples containing Aβ oligomers, lyophilized Aβ1-42 was dissolved in 10 mM NaOD then diluted 1:1 with 20 mM phosphate buffer (pH 7.4) and cocoa extracts or enriched fractions (10 mg/mL). The pH of each sample was measured with a Microelectrode (InLab Micro, Mettler Toledo, Columbus, OH, USA) and adjusted to pH 7.4 with NaOD and/or DCl. All pH values were corrected for the isotope effect. Experiments were run on an AVANCE III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H lock) cryogenic probe.

A basic sequence from the Bruker library was employed for the STD experiment.

A train of Gaussian-shaped pulses of 50 ms each was employed to saturate the protein envelope selectively; the total saturation time of the protein envelope was adjusted to the number of shaped pulses and set at 3 s. On- and off-resonance spectra were acquired in an interleaved mode with the same number of scans. The STD NMR spectrum was obtained by subtracting the on-resonance spectrum from the off-resonance spectrum.

3. Results and discussion

3.1. Cocoa extract preparation and NMR-based metabolic profiling

Cocoa beans of *Lavado* cocoa or *Natural* cocoa (the nib only or the whole bean, consisting of nib and shell) were finely ground under liquid nitrogen; commercial cocoa did not require grinding as it was bought as a powder. For the *Lavado* and *Natural* cocoa beans, the extracts for the nibs only were preferred to those from the whole bean because of the higher extraction yields.

The utility of a defatting step before extraction was verified by preparing cocoa extracts from defatted and not defatted powders. The defatting process led to a considerable reduction in NMR resonances of the fatty acids, obtaining clearer samples, without affecting the profiles of the other metabolites. Therefore, the protocol involving a defatting step before extraction was preferred.

Cocoa extracts were prepared according to different procedures: extraction in boiling water, extraction with water using a Soxhlet apparatus, extraction with methanol using a Soxhlet apparatus, hydro-alcoholic extraction with boiling water/methanol (8:2 v/v), hydro-alcoholic extraction with water (pH 4.5)/methanol 7:3, extraction with acetone/water/acetic acid (70:29.5:0.5), all according to already described protocols (see Materials and Methods section for experimental details and references). For each procedure, we calculated the extraction yield and examined the ^{1}H NMR metabolic profile (Supplementary Material – Fig. S1). We selected hydro-alcoholic extraction with boiling water/methanol (8:2 v/v) and extraction in water using a Soxhlet apparatus as the best-performing ones, based on both extraction yields and metabolic composition, and they were repeated in triplicate. An ultrafiltration procedure (Supplementary Material – Fig. S2) was used to separate the high- molecular-weight (> 3kDa, HMW) and low-molecular-weight (< 3kDa, LMW) fractions, using tangential flow ultrafiltration devices with a polyether-sulfone membrane and a 3 kDa molecular weight cut-off (MWCO). The LMW fraction was employed to simplify the resolution and assignment of the NMR spectra, especially of the spectral regions where the broad resonances of high-molecular-weight species overlapped those of important cocoa metabolites

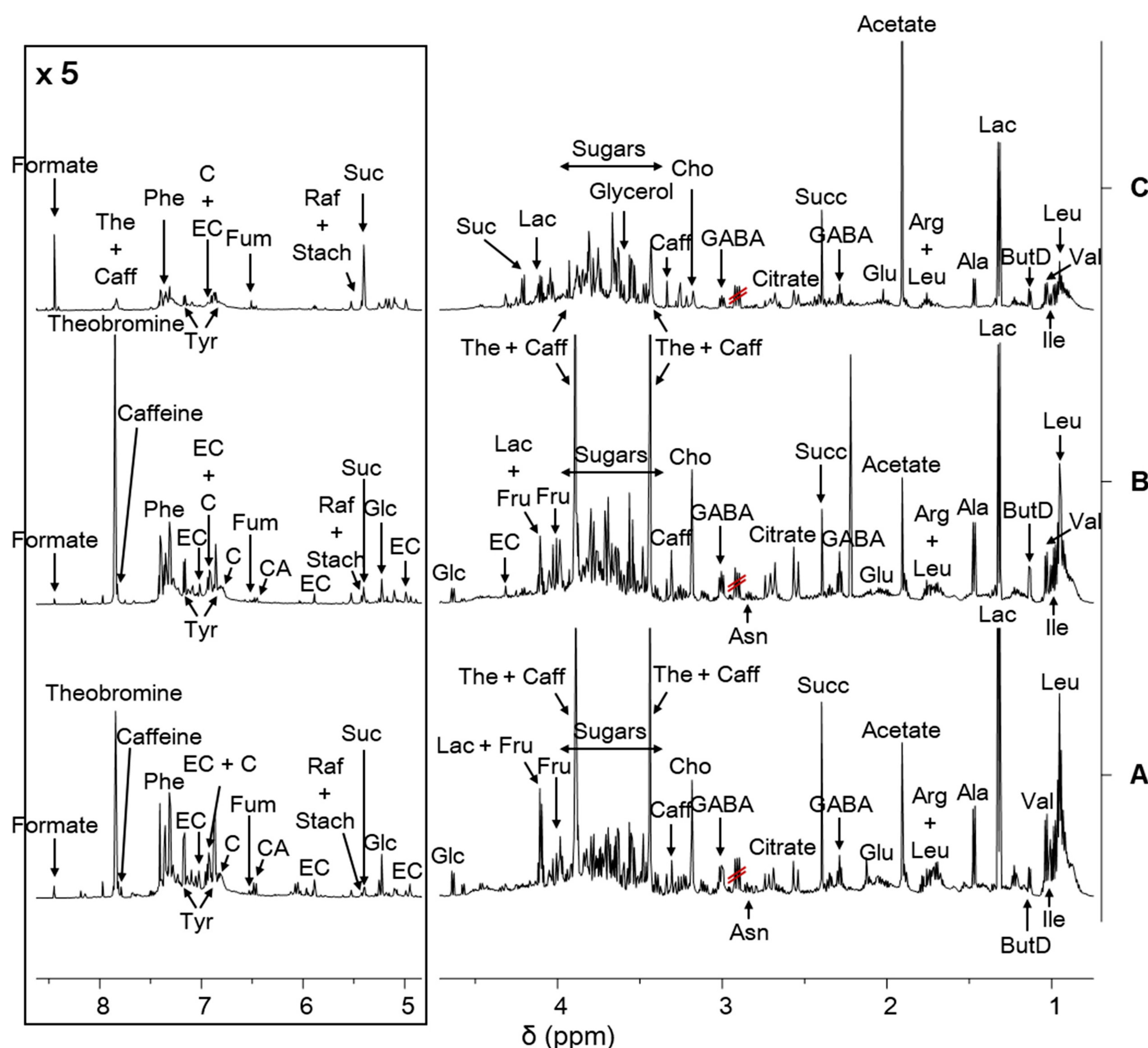

Fig. 1. ^{1}H NMR metabolic profiles of cocoa extracts (15 mg/mL, D_2O, pH 7) obtained in water with a Soxhlet apparatus from different cocoa beans: *Lavado* cocoa (A), *Natural* cocoa (B), commercial cocoa powder (C). Spectra were acquired at 25 °C and 600 MHz. Assignments of the resonances of the most important metabolites are reported (The, theobromine; Caff, caffeine; Phe, phenylalanine; Tyr, tyrosine; C, catechin; EC, epicatechin; Fum, fumarate; CA, caffeic acid derivatives; Raf, raffinose; Stach, stachyose; Suc, sucrose; Glc, glucose, Lac, lactate; Fru, fructose; Cho, choline; GABA, γ-aminobutyric acid; Asn, asparagine; Succ, succinate; Glu, glutamate; Arg, arginine; Leu, leucine; Ala, alanine; ButD, 2,3-butanediol; Val, valine; Ile, isoleucine).

(Supplementary Material – Fig. S2, 7.5–6.8 ppm, 4.3–3.2 ppm and 1.1–0.8 ppm).

Metabolites were identified by analysis of mono (^{1}H) and bi-dimensional (^{1}H,^{1}H-TOCSY, ^{1}H,^{13}C-HSQC) NMR spectra (Fig. 1 and Table 1; Supplementary Material – Figs. S3 and S4) and were in agreement with previous data on *Natural* cocoa extracts (Caligiani et al., 2010, 2014); however, to the best of our knowledge, NMR characterization of *Lavado* cocoa had not been done before and is reported here for the first time.

After manual identification of the compounds, we used a procedure previously set up by our group for the analysis of coffee and beer (Ciaramelli et al., 2019; Palmioli, Alberici, Ciaramelli, & Airoldi, 2020) to rapidly and semi-automatically identify and quantify the metabolites in all cocoa extracts. Briefly, specific libraries were built using the Simple Mixture Analysis (SMA) tool implemented in MestreNova 12.0.4 software. All these libraries are available as .exp files (Airoldi, Ciaramelli, & Palmioli, 2020).

SMA allows the simultaneous quantification of all the metabolites in a complex mixture and multiple samples can be analysed simultaneously. For each group of spectra, the plug-in output is presented by a table listing the metabolites identified and their concentrations. Assignment of metabolites can be rapidly checked by visual inspection of the spectra, where metabolite labels are automatically reported (Supplementary Material – Fig. S5).

This method considerably shortens the time required for spectra analysis and determination of metabolite concentrations. The manual procedure takes 30–45 min per spectrum, against the two minutes of the SMA-based approach. Table S1 (Supplementary Material) reports the extraction yields and the quantifications (mean concentrations as µg/mg of extract and corresponding standard deviations of three independent experiments) of metabolites in cocoa extracts, determined using the SMA plug-in.

Table 1
Assignments of metabolites in cocoa extracts. Chemical shifts reported refer to the hydro-alcoholic extract from *Natural* cocoa; slight chemical shift differences can be seen for the other extracts.

Metabolite	Assignment	^{1}H chemical shift	^{13}C chemical shift
Acetate	CH_3	1,91 (s)	25,87
Acetoin	CH_3 (5)	2,21 (s)	25,01
	CH_3 (4)	1,35 (d)	18,36
Alanine	CHα	3,77 (m)	53,29
	CH_3	1,47 (d)	22,79
Arginine	CHα	3,74 (m)	57,35
	CH_2δ	3,23 (m)	43,33
	CH_2β	1,89 (m)	30,43
	CH_2γ	1,72 (m)	29,19
Asparagine	CHα	3,99 (m)	54,23
	CH_2	2,83–2,93 (m)	37,40
Aspartate	CHα	3,9 (m)	54,91
	CH_2β	2,64–2,80 (m)	39,20
2,3-Butanediol	2 × CH	3,61–3,71 (m)	
	2 × CH_3	1,13 (t)	
Caffeine	CH ring (11)	7,85 (s)	146,12
	CH_3 (13)	3,91 (s)	36,24
	CH_3 (12)	3,49 (s)	32,68
	CH_3 (14)	3,31 (S)	30,77
Catechin	CH (19) (C2′H)	6,92 (m)	121,64
	CH (20), CH (21)	6,82 (m)	118,18
	CH (17)	6,04 (d)	99,82
	CH (18)	6,03 (d)	99,19
Choline	2 × CH_3	3,19 (s)	56,6
Citrate	CH_2 (2), CH_2 (6)	2,69 (d)–2,55 (d)	48,51
Epicatechin	CH (19) (C2′H)	7,02 (s)	117,12
	CH (20), CH (21)	6,92 (m)	118,88
	CH (18)	6,06 (m)	102,00
	CH (17)	6,04 (m)	95,50
	CH (8)	4,94 (s)	80,93
	CH (7)	4,31 (s)	68,52
Formate	O=C–H	8,45 (s)	
Fructose	CH (6 $_{eq}$) (pyranose)	4.02 (m)	73,6
	CH (4) (furanose)	4,10 (m)	73,5
	CH (4) (pyranose)	3,89 (dd)	72,6
	CH (3) CH (5) (furanose)	3,79 (m)	66,6
	CH (6) (furanose) + CH (1b), CH (6 eq) (pyranose)	3,70 (m)	65
	CH (1_b) (pyranose)	3,55 (m)	66,7
Fumarate	CH = CH	6,51 (s)	
GABA (γ-Aminobutyric acid)	CH_2 (4)	3,01 (t)	42,13
	CH_2 (2)	2,29 (t)	37,27
	CH_2 (3)	1,89 (p)	26,44
α-D-Glucose	CH (1)	5,22 (d)	94,95
	CH (2)	3,83 (m)	74,19
	CH_2 (6)	3,82–3,76 (m)	63,55
	CH (3)	3,7 (m)	75,58
	CH (5)	3,52 (m)	74,02
	CH (4)	3,4 (m)	72,44
β-D-Glucose	CH (1)	4,64 (d)	98,71
	CH_2 (6)	3,71–3,89 (m)	63,58
	CH (3), CH (5)	3,47 (m)	78,63
	CH (4)	3,41 (m)	72,44
	CH (2)	3,24 (m)	77
Glutamate	CHα	3,75 (m)	57,30
	CH_2γ	2,35 (m)	36,20
	CH_2β	2,07 (m)	29,52
Glycerol	CH	3,77 (m)	74,89
	2 × CH_2	3,55 (dd)-3,64 (dd)	65,3
Isoleucine	CHα	3,71 (d)	62,38
	CH	1,97 (m)	38,59
	CH_2	1,25–1,46 (m)	27,2
	CH_3 (7)	1,00 (d)	17,62
	CH_3 (6)	0,93 (t)	13,94
Lactate	CH	4,10 (q)	71,2
	CH_3	1,32 (d)	22,78
Leucine	CHα	3,72 (d)	56,28
	CH_2 + CH	1,71 (m)	CH 27,07; CH_2 42,70
	2 × CH_3	0,95 (t)	24,79
Malate	CH_2	2,74 (dd)–2,62 (dd)	
Phenylalanine	CH (7), CH (9)	7,41 (m)	131,93
	CH (8)	7,35 (m)	130,54
	CH (6), CH (10)	7,31 (d)	132,17
	CHα	3,98 (m)	58,86
	CH_2β	3,10 (dd)–3,27 (dd)	39,4
Succinate	2 × CH_2	2,39 (s)	37,08
Sucrose	CH (7) (Glc)	5,41 (d)	95
	CH (3) (Fru)	4,21 (d)	79,21
	CH (4) (Fru)	4,05 (t)	75,07
	CH (5) (Fru)	3,88 (m)	84,18
	CH (9) (Glc)	3,83 (m)	74,94
	CH 17 + 19	3,81 (m)	83,89
	CH (11) (Glc)	3,75 (t)	73,75
	CH (13) (Fru)	3,67 (s)	64,21
	CH (12) (Glc)	3,55 (dd)	73,77
	CH 1(0) (Glc)	3,47 (t)	72,12
Theobromine	CH ring (11)	7,86 (s)	146,51
	CH_3 (13)	3,9 (s)	36,23
	CH_3 (12)	3,44 (s)	31,8
Tyrosine	CH (7), CH (10)	7,17 (d)	133,6
	CH (6), CH (11)	6,86 (d)	118,66
	CHα	3,92 (m)	58,82
	CH_2β	3,04 (dd)–3,18 (dd)	38,21
Valine	CHα	3,60 (d)	63,3
	CH (2)	2,26 (m)	31,99
	CH_3 (6)	1,03 (d)	20,82
	CH_3 (3)	0,98 (d)	19,56

3.2. Cocoa extracts inhibit Aβ1-42 peptide aggregation and reduce Aβ1-42-induced neurotoxicity

Cocoa extracts (N1 – *Natural* cocoa extracted with Soxhlet in H_2O; N2 – *Natural* cocoa extracted with H_2O/MeOH 8:2; L1 – *Lavado* cocoa extracted with Soxhlet in H_2O; L2 – *Lavado* cocoa extracted with H_2O/MeOH 8:2; C1 – Commercial cocoa extracted with Soxhlet in H_2O; C2 – Commercial cocoa extracted with H_2O/MeOH 8:2) were tested by ThT assay (Hawe, Sutter, & Jiskoot, 2008) for their ability to inhibit the aggregation of Aβ1-42 peptide. Aβ1-42 peptide (2.5 μM) was co-incubated for 24 h at 37 °C with 15.6 μg/ml of extracts N1, N2, L1, L2, C1 and C2. This treatment strongly reduced the peptide aggregation in all the samples (Fig. 2A).

To assess the ability of cocoa extracts to reduce Aβ1-42 cytotoxicity, we co-treated the human neuroblastoma SH-SY5Y cell line for 24 h with Aβ oligomers and N1, N2, L1, L2, C1 and C2 extracts. Cell viability was reduced by 44% ($p < 0.001$ vs vehicle) after incubation of cells with 10 μM of Aβ1-42; this effect was antagonized by cocoa extracts added at a concentration of 62.5 μg/mL, leading to different increases in cell survival (N1 18.3%, N2 31.3%, L1 37.3%, L2 30%, C1 18.7% and C2 27.7%, $p < 0.001$ vs Aβ1-42 alone) (Fig. 2B). These data suggested that cocoa extracts reduce Aβ1-42 neurotoxicity and that this activity is exerted, at least partly, through the inhibition of Aβ oligomerization.

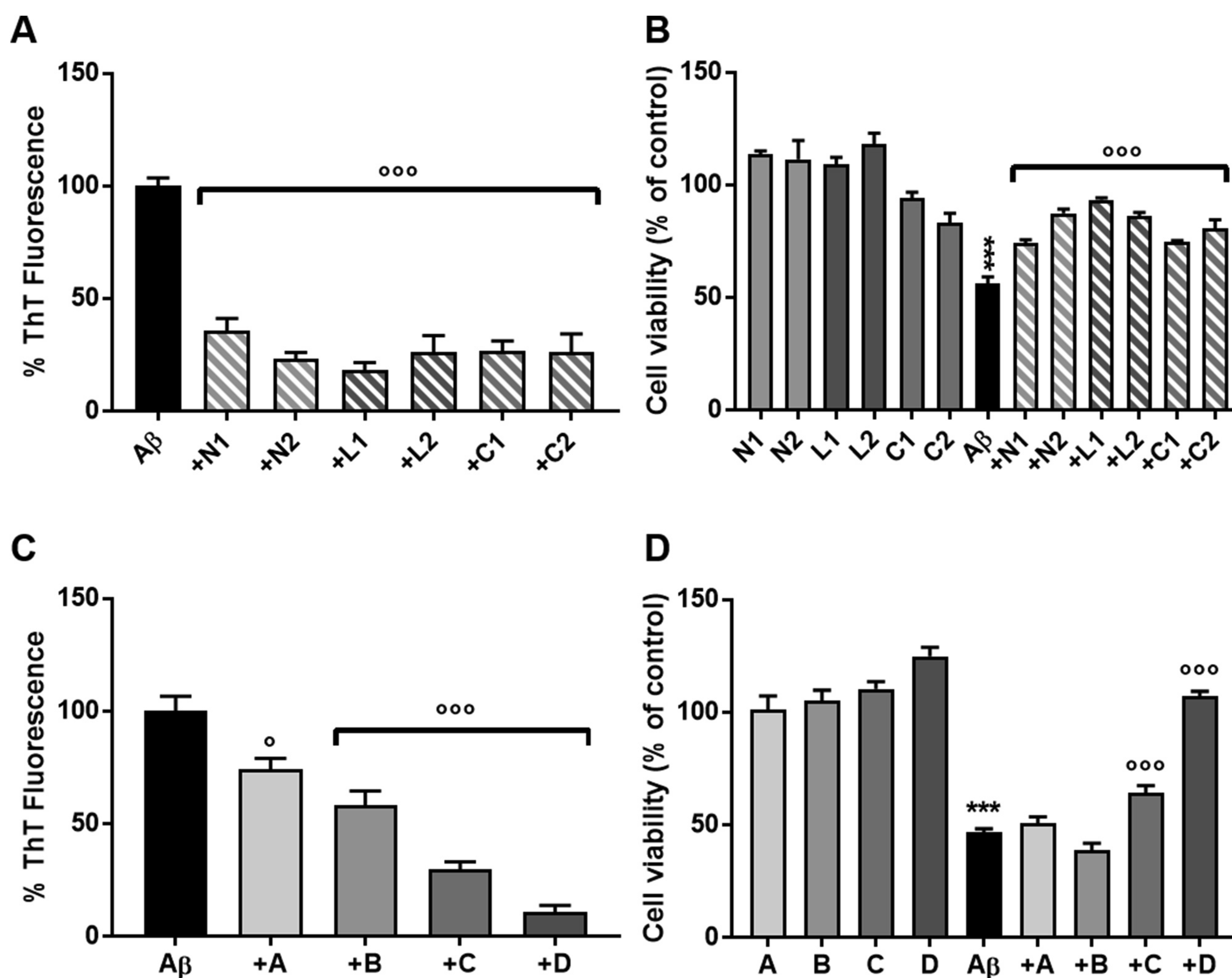

Fig. 2. A) Effects of cocoa extracts on Aβ1-42 aggregation. The ThT fluorescence assay was used to investigate the effects of co-incubation (24 h at 37 °C) of N1, N2, L1, L2, C1 and C2 extracts (15.6 μg/mL) on Aβ1-42 (2.5 μM) aggregation. Values are mean ± standard deviation of three replicates, after subtraction of the relative control solutions (extracts alone). °°°$p < 0.001$ vs Aβ1-42 alone. One-way ANOVA followed by Dunnett's multiple comparisons test. B) Effects of cocoa extracts on Aβ-induced neurotoxicity in the human neuroblastoma SH-SY5Y cell line. Cells were treated with 62.5 μg/mL of N1, N2, L1, L2, C1 or C2 with 10 μM Aβ1-42 for 24 h, and viability was determined with an MTT assay. The effect of each treatment (cocoa extracts or Aβ1-42 alone) on cell viability is also shown. Values are mean ± standard deviation of three replicates. One-way ANOVA followed by Dunnett's multiple comparison test: ***$p < 0.001$ vs vehicle, °°°$p < 0.001$ vs Aβ1-42 alone. C) Effects of L1 cocoa fractions on Aβ1-42 aggregation. The effects of co-incubation (24 h at 37 °C) of A, B, C and D fractions (12.5 μg/mL) on Aβ1-42 (2.5 μM) aggregation was determined with the ThT fluorescence assay. Values are mean ± standard deviation of three replicates, after subtraction of the relative control solutions (extracts alone). °$p < 0.05$, °°°$p < 0.001$ vs Aβ1-42 alone. One-way ANOVA followed by Dunnett's multiple comparisons test. D) Effects of L1 cocoa fractions on Aβ-induced neurotoxicity in the human neuroblastoma SH-SY5Y cell line. Cells were treated with 50 μg/mL of A, B, C and D fractions and 10 μM Aβ1-42 for 24 h, and viability was determined with an MTT assay. The effect of each treatment (cocoa extracts or Aβ1-42 alone) on cell viability is also shown. Values are mean ± standard deviation of three replicates. One-way ANOVA followed by Dunnett's multiple comparison test: ***$p < 0.001$ vs vehicle, °°°$p < 0.001$ vs Aβ1-42 alone. N1 – *Natural* cocoa extracted with Soxhlet in H_2O; N2 – *Natural* cocoa extracted with H_2O/MeOH 8:2; L1 – *Lavado* cocoa extracted with Soxhlet in H_2O; L2 – *Lavado* cocoa extracted with H_2O/MeOH 8:2; C1 – Commercial cocoa extracted with Soxhlet in H_2O; C2 – Commercial cocoa extracted with H_2O/MeOH 8:2.

On comparing the activity of the samples, *Lavado* extracts (L1 and L2) appeared the most effective. Visual inspection of the ^{1}H NMR profiles of extracts, with the comparison of metabolite concentrations, suggested that the most marked differences among extracts depended on their polyphenol content, particularly flavanols.

3.3. Cocoa extracts' antioxidant capacity

Treating the cells with different antioxidants can significantly affect proteomic changes due to Aβ-mediated oxidative stress in different cell lines – including SH-SY5Y, leading to decreased cell viability (Cheignon et al., 2018). We therefore compared the antioxidant capacity of cocoa extracts by spectrophotometry to verify any possible correlations between their ability to counteract oxidative stress and the MTT results (Fig. 2B).

To start with, UV–Vis absorption spectra (Fig. 3A) showed an intense absorption peak centred at 276 nm and a smaller broad absorption at 320 nm, probably associated respectively with monomeric catechins and other polyphenols. The antioxidant capacity in relation to the total polyphenols content and radical scavenging ability are reported in Fig. 3, panel **B** and **C**-**D**. There were no significant differences between the freeze-dried extracts, with the average around 140 mg of GAE /g and 1190 mmol.

Since *Lavado*, *Natural* and commercial cocoa extracts had comparable anti-oxidative properties, it would appear that their ability to counteract oxidative stress cannot explain their different potencies in preventing Aβ oligomer-induced cell damage (Fig. 2B).

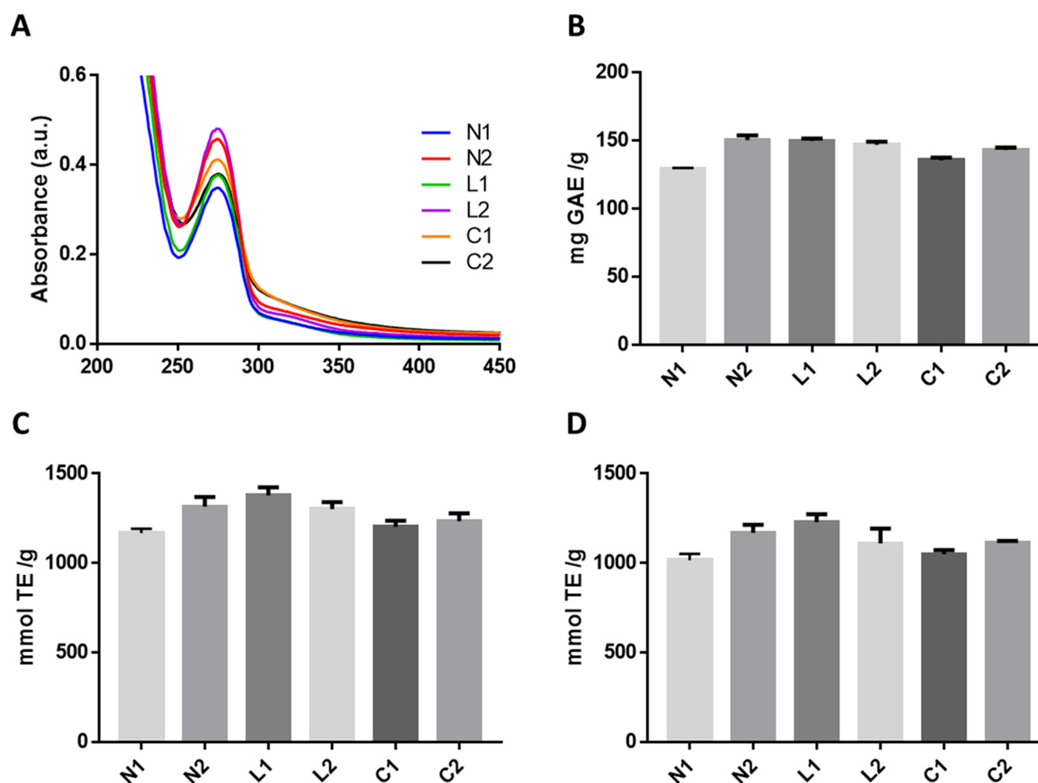

Fig. 3. A) UV–Vis absorption spectra of cocoa extracts dissolved at 50 μg/mL in MilliQ water. B) Total polyphenol content expressed as mg of gallic acid equivalent (GAE) per g of freeze-dried cocoa extract. C, D) Radical scavenging capacity (ABTS and DPPH assays respectively) expressed as mmol of Trolox equivalent (TE) per g of freeze-dried cocoa extract. Results are expressed as mean ± SD of triplicate measurements of two independent experiments. N1 – *Natural* cocoa extracted with Soxhlet in H_2O; N2 – *Natural* cocoa extracted with H_2O/MeOH 8:2; L1 – *Lavado* cocoa extracted with Soxhlet in H_2O; L2 – *Lavado* cocoa extracted with H_2O/MeOH 8:2; C1 – Commercial cocoa extracted with Soxhlet in H_2O; C2 – Commercial cocoa extracted with H_2O/MeOH 8:2.

3.4. Fractions of Lavado extracts enriched in flavanols inhibit Aβ1-42 peptide aggregation and reduce Aβ1-42-induced neurotoxicity

To verify whether the *Lavado* extracts' anti-amyloidogenic activity strongly correlates with their polyphenol content, they were fractionated by reverse-phase (RP) chromatography and the biological activity of the different fractions was tested.

Fractions of cocoa extracts enriched in different groups of molecules were obtained by RP C18 chromatography, using a Biotage® Isolera™ Prime system equipped with Spektra package (elution gradient: 98% H_2O → 100% MeOH). Their metabolic profiles were characterized by NMR spectroscopy. Fig. 4 reports the chromatographic profile (Fig. 4A) and the ^{1}H NMR spectra (Fig. 4B) of the enriched fractions obtained from *Lavado* cocoa extract L1 (Soxhlet in water); fraction E was not analyzed because it contained < 1 mg of product. The metabolites in the fractions were identified by NMR (assignments of the most important metabolites are reported in Fig. 4B-E) and quantified (Supplementary Material – Table S2) using the SMA plug-in of MestreNova Software, as previously described for the cocoa extracts.

Then, to identify the compounds, or their classes, responsible for the neuroprotective activity of cocoa extracts, we tested all the fractions for their biological activity. The ability of cocoa fractions to affect Aβ protein aggregation and Aβ-induced neurotoxicity was tested with thioflavin T (ThT), AFM and MTT assays. To verify the potential anti-amyloidogenic activity of L1 cocoa fractions, we assessed their effectiveness in inhibiting the aggregation of Aβ1-42 peptide with the ThT assay by incubating the peptide for 24 h at 37 °C with fractions A-D dissolved at a final concentration of 12.5 μg/mL (Fig. 2C). ThT fluorescence was 26% lower for fraction A ($p < 0.05$), 41.7% for B, 70.3% for C and 89.3% for D ($p < 0.001$), indicating significant inhibitory activity, particularly for fractions C and D.

AFM (Supplementary Material – Fig. S6) confirmed that cocoa fractions C and D interfered with the aggregation of Aβ1-42. When monomeric Aβ1-42 was incubated alone for 24 h at a final concentration of 2.5 μM, a family with protofibrillar structure of different sizes ranging in length from 0.1 to 2.0 μm formed (~80%) (Supplementary Material – Fig. S6**, A-E**). When the Aβ1-42 was incubated with cocoa fractions D and C amorphous material and a few globular and protofibrillar structures formed (Supplementary Material – Fig. S6, **D** and **I** for fraction C, Fig. S6**, E** and **L** for fraction D). In contrast, co-incubation with cocoa fraction B did not alter the kinetics of aggregation of the Aβ1-42, as evidenced by the formation of protofibrillar structures with the same sizes as Aβ1-42 alone (Supplementary Material – Fig. S6, **C** and **H**). Incubation with fraction B was only apparently able to reduce the aggregation of Aβ1-42. This co-incubation delayed the aggregation with the formation of a large family of small oligomers of different sizes within a range of 20–40 nm in diameter (~70%) (Supplementary Material – Fig. S6, **B**, **G** and **G** inset) that had toxic effects after treatment of SH-SY5Y cells (Fig. 2D).

The protective effect of L1 cocoa fractions against the toxicity induced by Aβ1-42 peptide was assessed in the MTT assay (Fig. 2D). Cotreatment of 10 μM Aβ1-42 with 50 μg/mL fractions for 24 h restored cell viability by 17.3% for fraction C (whose main constituent was theobromine, amounting to 12.3% w/w – Fig. 4B) and 60.3% for fraction D (whose most abundant components were flavanols – Fig. 4B); fractions A and B (mainly containing sugars, amino acids and other very polar metabolites – Fig. 4B) showed no effect.

3.5. Flavanols and theobromine in cocoa extracts interact with Aβ1–42 oligomers

STD NMR experiments were done to identify the *Lavado* extract

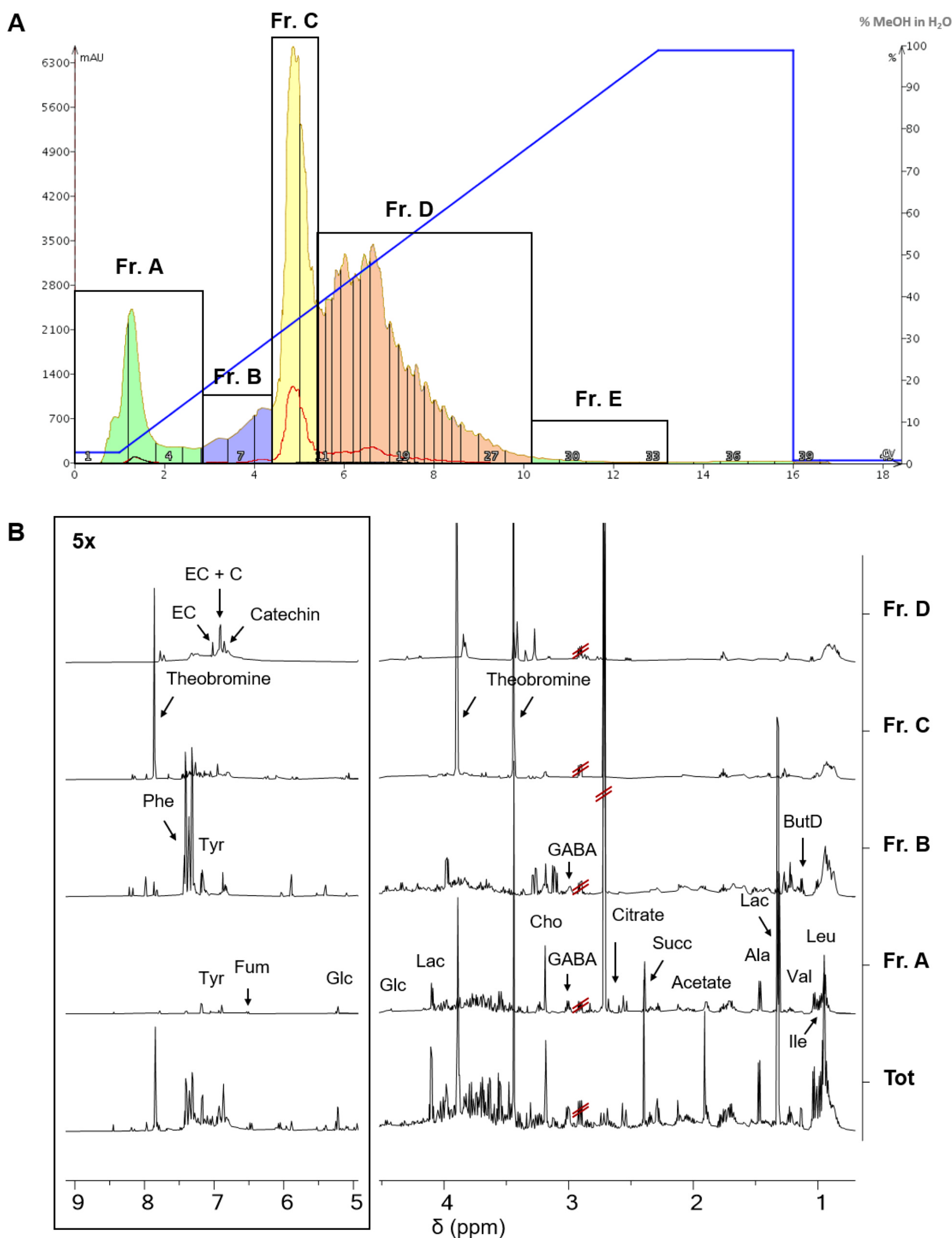

Fig. 4. Chromatographic profile (A) of the separation of *Lavado* cocoa extract L1 (Soxhlet in water) in enriched fractions, obtained by reverse phase C18 chromatography (elution gradient: 98% H_2O → 100% MeOH). (B) The ^{1}H NMR spectra of the chromatographic fractions A-D compared to the ^{1}H NMR profile of the total extract (Tot). ^{1}H NMR spectra were recorded on 10 mg/mL samples dissolved in D_2O, pH 7.0, 25 °C at 600 MHz. Assignments of the resonances of the most representative metabolites of each fraction are reported (Phe, phenylalanine; Tyr, tyrosine; C, catechin; EC, epicatechin; Fum, fumarate; Glc, glucose, Lac, lactate; Cho, choline; GABA, γ-aminobutyric acid; Succ, succinate; Leu, leucine; Ala, alanine; ButD, 2,3-butanediol; Val, valine; Ile, isoleucine). Assignments of the resonances of extract L1 (Tot) are reported in Fig. 1A.

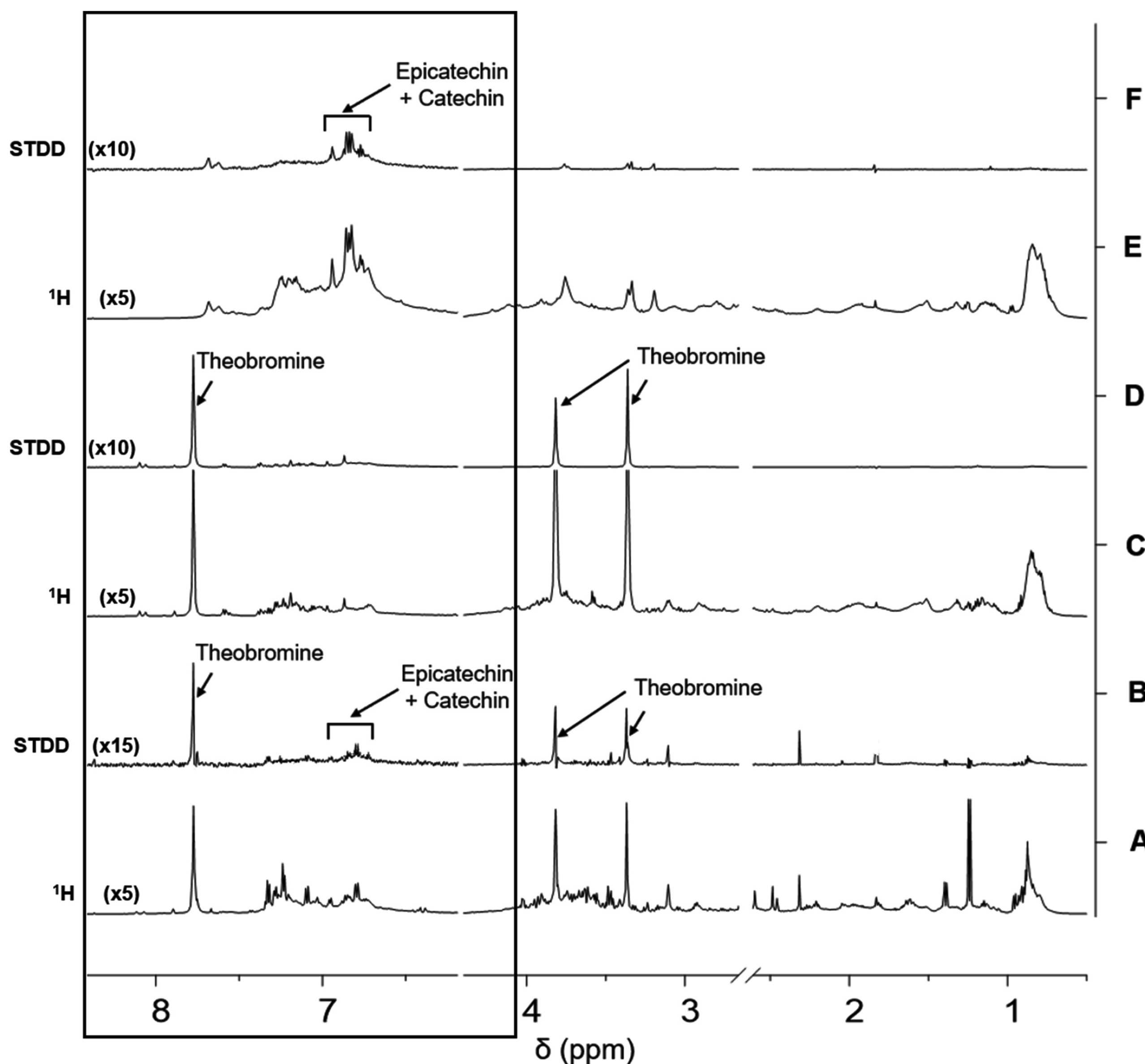

Fig. 5. ^{1}H NMR and STDD spectra on a mixture of *Lavado* cocoa extract (L1) (A and B), enriched fraction C of L1 (C and D) or enriched fraction D of L1 (E and F) (10 mg/mL) and Aβ1-42 protein (120 μM) in deuterated phosphate buffer, pH 7.4. STD spectra were acquired with 2048 scans and 2 s saturation time at 600 MHz, 25 °C. Different amplification factors (x5, x10, x15) of the aromatic regions were used to optimize visualization of the signals of interest in each spectrum.

components that interact directly with Aβ oligomers (Airoldi et al., 2013; Ciaramelli et al., 2018; Guzzi et al., 2017; Jesus et al., 2014; Palmioli et al., 2019). Spectra were acquired on mixtures containing Aβ oligomers and L1 (Fig. 5A and B), fraction C from L1, (Fig. 5C and D) or fraction D from L1, (Fig. 5E and F). A reference experiment on a sample containing only the crude extract L1, fraction C or fraction D was run under the same experimental conditions to verify true ligand binding (data not shown). The corresponding Saturation Transfer Double Difference spectra (STDD) (Airoldi, Giovannardi, La Ferla, Jimenez-Barbero, & Nicotra, 2011; Palmioli et al., 2020) were calculated (Fig. 5B, D and F) to estimate the STD effect due to the extract and fractions' component(s) directly binding to Aβ oligomers.

To ensure its oligomeric state, Aβ1–42 peptide was dissolved in aqueous phosphate buffer according to the procedure previously described (Airoldi et al., 2011). Selective saturation of some aliphatic protons of Aβ oligomers was achieved by irradiating the sample at −1.00 ppm (on-resonance frequency). If Aβ oligomers bind to other molecular entities (Aβ ligand(s)) in solution, magnetization is transferred from the receptor to the ligand(s) protons, so ligand(s) NMR signals can be detected in STDD spectra (Fig. 5, spectra **B**, **D** and **F**). Any signal from non-binding compounds is erased in the STDD spectrum, indicating that the molecule is not a ligand. The presence of theobromine (Fig. 5, spectra **B** and **D**) and the flavanols epicatechin and catechin resonances (Fig. 5, spectra **B** and **F**) in the STDD spectra is an unambiguous demonstration of the interaction of these compounds with Aβ1-42 oligomers.

Thus, molecular recognition experiments indicated that metabolites of cocoa extracts able to bind Aβ peptides, affecting both its aggregation and neurotoxicity, are flavanols, the most abundant being catechin and epicatechin, and theobromine.

3.6. Cocoa extracts do not potentiate autophagic pathways in human neuroblastoma SH-SY5Y cells under standard culture conditions

To verify whether the protective role of cocoa extracts seen in this study against Aβ aggregation and toxicity could, to some extent at least, be mediated by a potentiation of autophagy, we examined the expression of the key proteins of the main autophagic pathways,

macroautophagy and chaperone-mediated autophagy (CMA), involved in Aβ clearance. Human neuroblastoma SH-SY5Y cells were exposed for 24 h to 50 μg/mL cocoa extract L1 or fraction D (which had higher anti-amyloidogenic activity in the previous experiments reported), and protein levels of two macroautophagy (beclin-1 and LC3-II) and two CMA (LAMP2A and HSC70) effectors were measured by Western blot. Exposure to either extract L1 or the D fraction did not significantly alter the expression of any of the autophagy-related proteins (Supplementary Material – Fig. S7). Neither the total nor the D fraction from cocoa extracts stimulated the autophagic pathways involved in Aβ clearance, suggesting that the protective role of cocoa extracts demonstrated in this study is independent of any potentiation of autophagy in SH-SY5Y cells under standard culture conditions. It has in fact been reported in mouse liver cells under basal conditions that unroasted and roasted cocoa extracts do not activate macroautophagy (evaluated by analysis of Beclin-1 expression) (Arlorio et al., 2009).

The lack of effect of cocoa on autophagy suggests that flavanols, the main components of cocoa, are unable to potentiate this intracellular catabolic pathway, differently from the findings of our group in the same cell line after exposure to coffee extracts and melanoidins, which did induce autophagy (Ciaramelli et al., 2018).

4. Conclusions

We describe here, for the first time, the ^{1}H NMR characterization of *Lavado* cocoa's metabolic profile. Starting by comparing its chemical composition with those of *Natural* and commercial varieties, we investigated the anti-amyloidogenic activity of the different cocoa extracts and their main molecular constituents. We characterized the extracts' ability to target Aβ oligomers and prevent their neurotoxicity and their action on chaperone-mediated autophagy and macroautophagy, dissecting the neuroprotective effects of extracts and their main constituents. *Lavado* extracts had the most potent biological activity, which correlated with the highest levels of the flavanols catechin and epicatechin and theobromine.

By fractionation of the *Lavado* cocoa extract and advanced NMR-based ligand-receptor studies, we demonstrated that flavanols and theobromine are Aβ-oligomer ligands, suggesting that their ability to hinder peptide fibrillation and neurotoxicity depends on direct interaction with the target. This finding indicates different behavior for theobromine and caffeine, another methylxanthine very abundant in nature. Our previous investigation on coffee extracts (Ciaramelli et al., 2018) did clearly indicate that caffeine does not have the same biological activities as seen here for theobromine. Moreover, the lack of effect of the cocoa extract on autophagy amounts to another significant difference from coffee extract, suggesting that cocoa anti-AD activity is mainly due to its components (catechins and theobromine in particular) being able to act on the aggregation and toxicity of the Aβ peptides.

Absorption and bioavailability of polyphenols and flavonoids from foods is a complex process influenced by several factors, such as food processing and food matrix composition, as well as host-related factors (Neilson & Ferruzzi, 2011). Flavan-3-ols are the main dietary flavonoids, and monomeric catechins and epicatechins from cocoa are rapidly absorbed by the human intestine and peak in plasma within 2–3 h, returning to baseline 6 h after ingestion (Rusconi & Conti, 2010; Vitaglione et al., 2013). Besides, a recent clinical trial study reported that methylxanthines such as theobromine affect the absorption of epicatechins, enhancing the vascular effects commonly ascribed to cocoa flavanols (Sansone et al., 2016). In the light of this evidence, regular use of polyphenol-rich dietary sources and well-characterized functional food supplements producing circulating bioactive molecules, including flavanols, might possibly be preventive through the so-called "peripheral sink-effect" mechanism (Zhang & Lee, 2011). As Aβ peptides in the brain and periphery are in equilibrium, the removal of Aβ in the periphery, followed by its passive diffusion down a concentration gradient, would lower Aβ in the brain. Peripherally administered Aβ-binding agents can promote this clearance.

In addition, the ability of both theobromine (Sugimoto, Katakura, Matsuzaki, Ohno-Shosaku, Yachie, & Shido, 2016) and catechins to cross the blood/brain barrier (Faria et al., 2011), possibly reaching the central nervous system, has been reported, suggesting that their anti-amyloidogenic effect might be exerted in both the blood and the brain.

The present results suggest that cocoa extracts, particularly those with a high content of flavanols and theobromine, could be usefully exploited to develop nutraceuticals and functional foods for the prevention of AD and other NDs. *Lavado* extracts, which have the highest anti-amyloidogenic activity, appear to be the most promising.

The biochemical events underlying the onset of these NDs take place several years before their clinical manifestations (McDade & Bateman, 2017), suggesting the use of bioactive molecules to prevent amyloid aggregation as a promising prophylactic strategy. Regular intake in the diet of natural compounds capable of interfering with toxic Aβ oligomers might well be very effective.

CRediT authorship contribution statement

Carlotta Ciaramelli: Methodology, Validation, Formal analysis, Data curation, Writing - original draft. **Alessandro Palmioli:** Methodology, Validation, Formal analysis, Investigation, Writing - review & editing. **Ada De Luigi:** Investigation, Formal analysis. **Laura Colombo:** Investigation, Formal analysis. **Gessica Sala:** Investigation, Formal analysis. **Mario Salmona:** Resources, Funding acquisition. **Cristina Airoldi:** Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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References

Airoldi, C., Ciaramelli, C., & Palmioli, A. (2020). SMA libraries for metabolite identification and quantification in cocoa extracts. *Mendeley Data, 1*.

Airoldi, C., Colombo, L., Manzoni, C., Sironi, E., Natalello, A., Doglia, S. M., ... Salmona, M. (2011). Tetracycline prevents Aβ oligomer toxicity through an atypical supramolecular interaction. *Organic & Biomolecular Chemistry, 9*(2), 463–472.

Airoldi, C., Giovannardi, S., La Ferla, B., Jimenez-Barbero, J., & Nicotra, F. (2011). Saturation transfer difference NMR experiments of membrane proteins in living cells under HR-MAS conditions: The interaction of the SGLT1 co-transporter with its ligands. *Chemistry, 17*(48), 13395–13399.

Airoldi, C., Sironi, E., Dias, C., Marcelo, F., Martins, A., Rauter, A. P., ... Jimenez-Barbero, J. (2013). Natural compounds against Alzheimer's disease: Molecular recognition of Aβ1–42 peptide by salvia sclareoides extract and its major component, rosmarinic

acid, as investigated by NMR. *Chemistry – An Asian Journal, 8*(3), 596–602.

Amigoni, L., Stuknytė, M., Ciaramelli, C., Magoni, C., Bruni, I., De Noni, I., ... Palmioli, A. (2017). Green coffee extract enhances oxidative stress resistance and delays aging in Caenorhabditis elegans. *Journal of Functional Foods, 33*, 297–306.

Arlorio, M., Bottini, C., Travaglia, F., Locatelli, M., Bordiga, M., Coïsson, J. D., ... Tessitore, L. (2009). Protective activity of Theobroma cacao L. phenolic extract on AML12 and MLP29 liver cells by preventing apoptosis and inducing autophagy. *Journal of Agriculture and Food Chemistry, 57*(22), 10612–10618.

Arlorio, M., Locatelli, M., Travaglia, F., Coïsson, J.-D., Grosso, E. D., Minassi, A., ... Martelli, A. (2008). Roasting impact on the contents of clovamide (N-caffeoyl-L-DOPA) and the antioxidant activity of cocoa beans (Theobroma cacao L.). *Food Chemistry, 106*(3), 967–975.

Caligiani, A., Acquotti, D., Cirlini, M., & Palla, G. (2010). 1H NMR study of fermented cocoa (Theobroma cacao L.) beans. *Journal of Agricultural and Food Chemistry, 58*(23), 12105–12111.

Caligiani, A., Palla, L., Acquotti, D., Marseglia, A., & Palla, G. (2014). Application of 1H NMR for the characterisation of cocoa beans of different geographical origins and fermentation levels. *Food Chemistry, 157*, 94–99.

Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C., & Collin, F. (2018). Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biology, 14*, 450–464.

Ciaramelli, C., Palmioli, A., & Airoldi, C. (2019). Coffee variety, origin and extraction procedure: Implications for coffee beneficial effects on human health. *Food Chemistry, 278*, 47–55.

Ciaramelli, C., Palmioli, A., De Luigi, A., Colombo, L., Sala, G., Riva, C., ... Airoldi, C. (2018). NMR-driven identification of anti-amyloidogenic compounds in green and roasted coffee extracts. *Food Chemistry, 252*, 171–180.

Cimini, A., Gentile, R., D'Angelo, B., Benedetti, E., Cristiano, L., Avantaggiati, M. L., ... Desideri, G. (2013). Cocoa powder triggers neuroprotective and preventive effects in a human Alzheimer's disease model by modulating BDNF signaling pathway. *Journal of Cellular Biochemistry, 114*(10), 2209–2220.

Dubner, L., Wang, J., Ho, L., Ward, L., & Pasinetti, G. M. (2015). Recommendations for development of new standardized forms of cocoa breeds and cocoa extract processing for the prevention of Alzheimer's disease: Role of cocoa in promotion of cognitive resilience and healthy brain aging. *Journal of Alzheimer's Disease, 48*, 879–889.

Faria, A., Pestana, D., Teixeira, D., Couraud, P.-O., Romero, I., Weksler, B., ... Calhau, C. (2011). Insights into the putative catechin and epicatechin transport across blood-brain barrier. *Food & Function, 2*(1), 39–44.

Guzzi, C., Colombo, L., Luigi, A. D., Salmona, M., Nicotra, F., & Airoldi, C. (2017). Flavonoids and their glycosides as anti-amyloidogenic compounds: Aβ1–42 interaction studies to gain new insights into their potential for Alzheimer's disease prevention and therapy. *Chemistry – An Asian Journal, 12*(1), 67–75.

Haass, C., & Selkoe, D. J. (2007). Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid β-peptide. *Nature Reviews Molecular Cell Biology, 8*(2), 101–112.

Hawe, A., Sutter, M., & Jiskoot, W. (2008). Extrinsic fluorescent dyes as tools for protein characterization. *Pharmaceutical Research, 25*(7), 1487–1499.

Heo, H. J., & Lee, C. Y. (2005). Epicatechin and catechin in cocoa inhibit amyloid β protein induced apoptosis. *Journal of Agricultural and Food Chemistry, 53*(5), 1445–1448.

Hurst, W. J., Krake, S. H., Bergmeier, S. C., Payne, M. J., Miller, K. B., & Stuart, D. A. (2011). Impact of fermentation, drying, roasting and Dutch processing on flavan-3-ol stereochemistry in cacao beans and cocoa ingredients. *Chemistry Central Journal, 5*(1), 53.

Jesus, A. R., Dias, C., Matos, A. M., de Almeida, R. F. M., Viana, A. S., Marcelo, F., ... Rauter, A. P. (2014). Exploiting the therapeutic potential of 8-β-d-glucopyranosylgenistein: synthesis, antidiabetic activity, and molecular interaction with islet amyloid polypeptide and amyloid β-peptide (1–42). *Journal of Medicinal Chemistry, 57*(22), 9463–9472.

Magrone, T., Russo, M. A., & Jirillo, E. (2017). Cocoa and dark chocolate polyphenols: from biology to clinical applications. *Frontiers in Immunology, 8*(677), 677.

Manzoni, C., Colombo, L., Messa, M., Cagnotto, A., Cantù, L., Del Favero, E., & Salmona, M. (2009). Overcoming synthetic Aβ peptide aging: A new approach to an age-old problem. *Amyloid, 16*(2), 71–80.

Marseglia, A., Acquotti, D., Consonni, R., Cagliani, L. R., Palla, G., & Caligiani, A. (2016). HR MAS 1H NMR and chemometrics as useful tool to assess the geographical origin of cocoa beans – Comparison with HR 1H NMR. *Food Research International, 85*, 273–281.

McDade, E., & Bateman, R. J. (2017). Stop Alzheimer's before it starts. *Nature News, 547*(7662), 153.

Nehlig, A. (2013). The neuroprotective effects of cocoa flavanol and its influence on cognitive performance. *British Journal of Clinical Pharmacology, 75*(3), 716–727.

Neilson, A. P., & Ferruzzi, M. G. (2011). Influence of formulation and processing on absorption and metabolism of flavan-3-ols from tea and cocoa. *Annual Review of Food Science and Technology, 2*(1), 125–151.

Palmioli, A., Alberici, D., Ciaramelli, C., & Airoldi, C. (2020). Metabolomic profiling of beers: Combining 1H NMR spectroscopy and chemometric approaches to discriminate craft and industrial products. *Food Chemistry, 327*, Article 127025.

Palmioli, A., Bertuzzi, S., De Luigi, A., Colombo, L., La Ferla, B., Salmona, M., ... Airoldi, C. (2019). bioNMR-based identification of natural anti-Aβ compounds in Peucedanum ostruthium. *Bioorganic Chemistry, 83*, 76–86.

Palmioli, A., Ceresa, C., Tripodi, F., La Ferla, B., Nicolini, G., & Airoldi, C. (2020). On-cell saturation transfer difference NMR study of Bombesin binding to GRP receptor. *Bioorganic Chemistry, 99*, Article 103861.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9), 1231–1237.

Rusconi, M., & Conti, A. (2010). Theobroma cacao L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacological Research, 61*(1), 5–13.

Sansone, R., Ottaviani, J. I., Rodriguez-Mateos, A., Heinen, Y., Noske, D., Spencer, J. P., ... Heiss, C. (2016). Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: Randomized, double-masked controlled studies. *The American Journal of Clinical Nutrition, 105*(2), 352–360.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*(4), 1202–1205.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Sironi, E., Colombo, L., Lompo, A., Messa, M., Bonanomi, M., Regonesi, M. E., ... Airoldi, C. (2014). Natural compounds against neurodegenerative diseases: molecular characterization of the interaction of catechins from green tea with Aβ1–42, PrP106–126, and ataxin-3 oligomers. *Chemistry, 20*(42), 13793–13800.

Sugimoto, N., Katakura, M., Matsuzaki, K., Ohno-Shosaku, T., Yachie, A., & Shido, O. (2016). Theobromine, the primary methylxanthine found in Theobroma cacao, can pass through the blood-brain barrier in mice. 30(S1), lb635–lb635.

Vitaglione, P., Barone Lumaga, R., Ferracane, R., Sellitto, S., Morelló, J. R., Reguant Miranda, J., ... Fogliano, V. (2013). Human bioavailability of flavanols and phenolic acids from cocoa-nut creams enriched with free or microencapsulated cocoa polyphenols. *British Journal of Nutrition, 109*(10), 1832–1843.

Wang, J., Varghese, M., Ono, K., Yamada, M., Levine, S., Tzavaras, N., ... Pasinetti, G. M. (2014). Cocoa extracts reduce oligomerization of amyloid-β: Implications for cognitive improvement in Alzheimer's disease. *Journal of Alzheimer's Disease, 41*, 643–650.

Zhang, Y., & Lee, D. H. S. (2011). Sink hypothesis and therapeutic strategies for attenuating Aβ levels. *The Neuroscientist, 17*(2), 163–173.