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Identification of the thistle milk component Silibinin(A) and Glutathione-disulphide as potential inhibitors of the pancreatic lipase: Potential implications on weight loss

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ABSTRACT

Peripheral targets like pancreatic-lipase appear to be the most suitable pharmacological alternative for obesity, as with orlistat, although its adverse effects limit its use. Therefore, the aim of this work was to identify new natural compounds able to inhibit pancreatic-lipase in an *in vitro* model. The DrugBank database was used to perform docking calculations. The best fitting-score compounds were further evaluated *in vitro*. Our data revealed that glutathione-disulphide (GSSG) and silibinin(A) inhibit pancreatic-lipase. This was confirmed by measuring hydrolysis in an emulsion model, obtaining that the suppression of lipid digestion by silibinin(A) was higher than that of GSSG and close to the effect of orlistat. Combined analysis established the existence of different inhibition mechanisms for each compound. In summary, silibinin(A) and GSSG inhibited pancreatic-lipase and, therefore, may be served as promise natural compounds to face with obesity. Further studies comprise the next step to fully validate the suitability of these compounds.

1. Introduction

Although many factors are implied on obesity development, an excess of energy intake, especially in the form of dietary fat, and a sedentary lifestyle are still considered as the key factors for its development (Heymsfield & Wadden, 2017). The problem of an excess of body fat is that those subjects are more exposed to the development of related diseases such as cardiovascular diseases and type 2-diabetes; therefore, apart from specific situations like the current COVID-19 epidemic, obesity and its consequences are still the main cause of mortality in the world (Kass, Duggal, & Cingolani, 2020). Ultimately, a caloric intake greater than energy expenditure origins the accumulation of body fat and, consequently, obesity. As a result, the intake of high-palatable and high-fat food may prone subjects to increase their body fat (Gadde, Martin, Berthoud, & Heymsfield, 2018). Unfortunately, the food industry adds fatty acids in their commercial foods because it provides better organoleptic and tasted sensations for consumers (Zabetakis, 2013).

At present, the gold therapy for obesity treatment is through modification of lifestyle habits, reducing energy intake through hypocaloric diets and increasing energy expenditure. Unfortunately, the effectiveness of this intervention is quite modest, especially at the long-term. In several situations, in addition to conventional therapy, some drugs are administered to reduce caloric intake and to improve dietary treatment performance (Greenway et al., 2010). There are several FDA approved drug for obesity treatment: on the one hand, there are central-acting agents like lorcaserin, phentermine/topiramato, and naltrexone/bupropion, which are useful but are usually related with several adverse

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effects, including seizures, serotonin toxicity, mood disorders and even memory loss (Kim, 2016). On the other hand, peripheral-acting agents like Liraglutide or orlistat have been also evidenced as effective anti-obesity agents. Orlistat is the best-selling drug worldwide, and it is the unique drug allowed in adolescents for obesity treatment; its efficiency is similar to other drugs, but it is more secure since undesirable side effects are related to gastro-intestinal disorders like fecal urgency, fecal incontinence, flatus and oily spotting, which often leads to discontinuation of therapy (Franson & Ro, 2000). It seems evident that the most suitable drug-therapy for obesity treatment might be through a similar mechanism to that of Orlistat, but with higher effectiveness and lower side-effects. In this regard, novel High-throughput screening techniques can allow us to find molecules that meet these characteristics.

Nevertheless, to evaluate the effectiveness of potential inhibitors of pancreatic lipase, like orlistat, it is necessary to understand that the substrates of pancreatic lipase (such as long-chain triacylglycerols, tributyrin, etc) are water-insoluble molecules, while the pancreatic lipase is water soluble. In this way, the pancreatic lipase is adsorbed onto the oil–water interface of emulsion droplets to hydrolyse triacylglycerol into free fatty acids (FFA) and monoglycerides, which will be later absorbed in the small intestine (Wilde & Chu, 2011). Therefore, lipolysis occurs at the oil–water interface of emulsified substrates and as consequence, the structure and composition of the interface is essential for pancreatic lipase activity (Li et al., 2018; Rahim, Takahashi, & Yamaki, 2015). Inhibition of lipase action can be originated by adsorption, conformational changes, enzymatic processes and desorption of the lipolysis products. Potential lipase inhibitors with surface active properties could compete for the oil–water interface impeding lipase activity. (Delorme et al., 2011). Hence, the efficacy of potential lipase inhibitors and the specific events taking place at the interface ultimately can be rapidly assessed by monitoring changes in the interfacial tension of isolated interfaces (Del Castillo-Santaella et al., 2015), which allow quantifying the inhibition in a more realistic scenario (Maldonado-Valderrama, 2019).

In this work, silibinin(A) and Glutathione-disulphide were selected by virtual screening and docking assays. Silibinin(A) is a flavonolignan extracted from milk thistle (*Onopordum acanthium*), which has been classically used for the treatment of fatty liver disorders (Singh, Gu, & Agarwal, 2008), while Glutathione-disulphide (GSSG) is the oxidized form of glutathione (GSH), an important natural antioxidant used as dietary supplement (Allen & Bradley, 2011).

The objective of this study was first to screen, through virtual screening, a library of compounds as potential inhibitors of pancreatic lipase. In this regard, the docking studies revealed that silibinin(A) and GSSG could inhibit the pancreatic lipase. In the next step, the inhibition of pancreatic lipase was measured in different levels: in solution, at oil-water interface and in emulsion. The interfacial rapid assessment previously developed (Del Castillo-Santaella et al., 2015) was further validated through the quantification of the percentage of FFA upon lipolysis of an emulsion model, which confirmed the *in silico* outcomes. Comparison of different methodologies provided complementary information allowing to establish the validity and limitations in order to obtain a valid model representative of the real situation.

2. Material and methods

2.1. Materials

Lipase from porcine pancreas (Type II, 100–400 units/mg) protein purchased from Sigma-Aldrich® (cat n. L3126), stored at 4 °C following manufacturer's instructions. Silibinin(A) was acquired from Sigma (S0417) and was stored at −18C and L-Glutathione oxidized from Sigma (G4376) was stored at −4C (Fig. 1). Tributyrin was purchased from Sigma-Aldrich® (W222305) and stored at room temperature. Highly refined olive oil (Sigma-Aldrich®, cat n. 01514) was purified with Florisil® resins (Fluka, 60–10 mesh, cat n. 46385) prior to be used following the procedure previously described (Del Castillo-Santaella et al., 2015; Maldonado-Valderrama, Torcello-Gómez, Del Castillo-Santaella, Holgado-Terriza, & Cabrerizo-Vílchez, 2015).

Titration buffer was prepared in 14.5 mL of TRIS-hydroxymethylaminomethane (36 mg/l), NaCl (9000 mg/l), $CaCl_2$ (200 mg/l), Sodium Taurodeoxycholate (2080 mg/l) and stored at 4 °C. Duodenal buffer was prepared in 2 mM BIS-TRIS (Sigma-Aldrich®, 14879), 0.15 M NaCl, 0.002 M $CaCl_2$, pH 7.0, according to the standardized *in vitro*

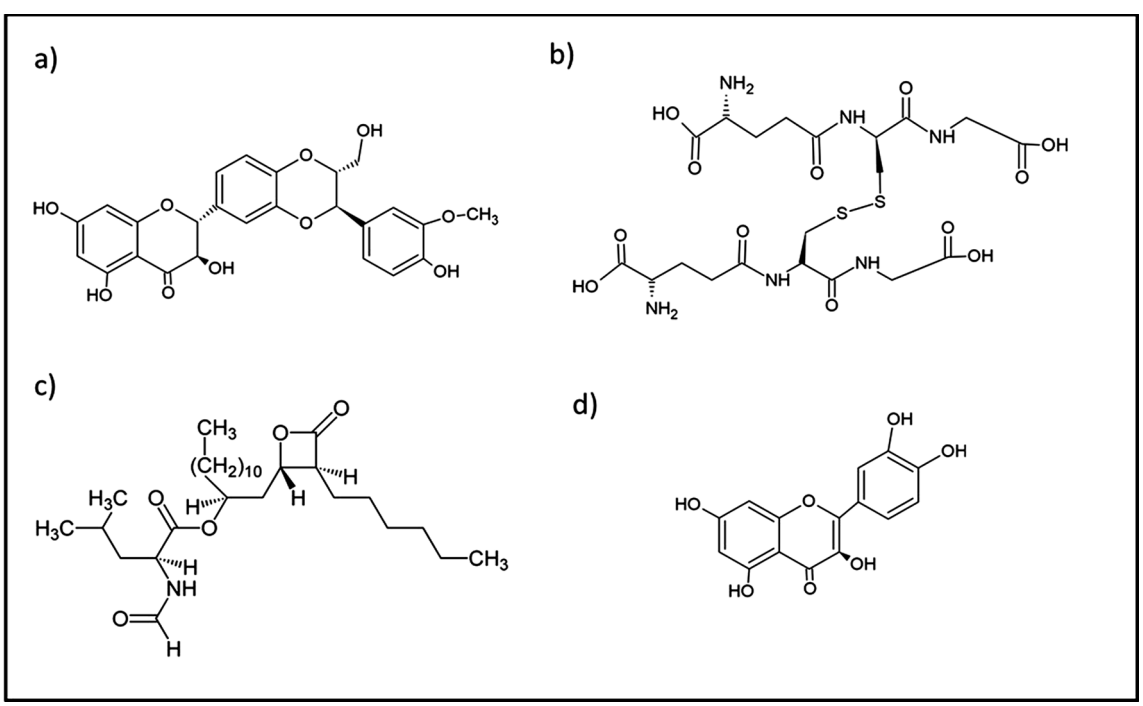

Fig. 1. The chemical structures of (a) Silibinin(A), (b) Glutathione-disulphide (GSSG), (c) Orlistat (Xenical®) and (d) Quercetin. ACD/ChemSketch software was used.

digestion method for food (Minekus et al., 2014). Lipase samples (7.8 g/l) were prepared immediately before use in the duodenal buffer with Bile salts (5 mg/ml) for microbiology (Sigma-Aldrich®, B8756). Inhibitors were added to this mixture and filtered before use with Millex® filters (0.1 μm PDVF).

Orlistat concentration was fixed at 0.44 mM in methanol (5.2% final concentrations). Silibinin(A) was dissolved in acetone and L-Glutathione oxidized was dissolved in duodenal buffer. Both inhibitors were tested at concentration 1.8 and 1.4 mM relevant to physiological studies (Del Castillo-Santaella et al., 2015). Lipase activity was measured with the same proportion of methanol or acetone as controls.

Ultrapure water, cleaned using a Milli-Q water purification system (0.054 μS), was used for the preparation of buffer solutions. All glassware was washed with 10% Micro-90 cleaning solution and exhaustively rinsed with tap water, isopropanol, deionized water, and ultrapure water in this sequence. All other chemicals used were of analytical grades and used as received.

2.2. Virtual screening

The first step to carry out this objective was the selection of an adequate compound database to conduct a virtual screening analysis. In this regard, the last release of DrugBank (version 5.1.4), which contains 13,574 drug entries including 2632 approved small molecule drugs, 1377 approved biologics (proteins, peptides, vaccines, and allergenics), 131 nutraceuticals and over 6375 experimental (discovery-phase) drugs, was employed, allowing a comprehensive search for potential lipase inhibitor compounds, with the advantage that they have already been tested (or are being tested) in humans, so that potential hits could be used for use in humans more quickly (Wishart et al., 2017). More important, this database been used successfully for the repurposing of drugs orphan diseases (Govindaraj, Naderi, Singha, Lemoine, & Brylinski, 2018), which confirms the suitability of our procedure.

All compounds deposited in DrugBank were set up for docking simulations by using AmberTools (AMBER 2017, University of California, San Francisco) (Case et al., 2017). Molecular parameters were calculated by removing salts and neutralizing their protonation state, computing partial charges by MMFF94 force field, adding hydrogen atoms and minimizing energies (default parameters) (Halgren, 1995).

The crystal structure of pancreatic lipase (Protein Data Bank code 1LPB) was used to build up the protein model system (Egloff et al., 1995). At an early stage, bond orders were assigned, hydrogens were added, and cap termini were included with the Protein Preparation Wizard module as implemented in Maestro (Schrödinger Release 2019–4: Maestro, Schrödinger, LLC, New York, USA) (Madhavi Sastry, Adzhigirey, Day, Annabhimoju, & Sherman, 2013). Protonation states of all side chains were subsequently defined using PROPKA3.1. Partial charges over all atoms were finally assigned within the AMBER99 force field scheme as implemented in AmberTools. Docking simulations were performed with Lead Finder software v1.1.20 (Stroganov, Novikov, Stroylov, Kulkov, & Chilov, 2008). All docking parameters were set to default for the calculations. The best ranked docking score posed for every compound was retained for further analysis.

2.3. Pancreatic lipase activity

2.3.1. The pHStat method

This method was carried out to evaluate the pancreatic lipase activity using tributyrin as substrate and titration buffer. The free fatty acids released by the lipase were titrated at a constant pH 8.0 by sodium hydroxide (NaOH, 0.01 N) perfusion and 37 °C. 0.5 mL of tributyrin were mixed with the titration buffer described above (Carriere, Barrowman, Verger, & René, 1993). Swine pancreatic lipase was also added at 50 μg. The inhibitors used in this method were: Orlistat (0.44 mM), GSSG (1.4 mM) and silibinin(A) (1.8 mM).

The experiment is based on measuring lipase activity alone and in co-administration with the potential inhibitors and determining the volume of NaOH needed to maintain a constant pH 8.0, in each case. Then, the enzymatic activity (U/mg in dry weight), is calculated by equation (1).

$$\frac{Units}{mgpowder} = \frac{R(NaOH) \times 1000}{v \times [E]} \tag{1}$$

where, R (NaOH) represented the amount of μmol used per minute, which is equal to the amount of μmol of fatty acids released per minute. The volume (v) of enzyme was expressed in μL, having used 50 μL for all the assays, and [E] is the enzymatic concentration (mg in dry weight/ml), which was 1 mg/ml.

2.3.2. Emulsion formation

Emulsions were formed by mixture of olive oil (1 g) with aqueous phase consisting of phosphate buffer (1.13 mM, pH 7.0) and 25% Tween-20 as model surfactant. First, the surfactant and buffer mixture was beaten in a Heidolph DIAX900 (potential 6, 1 min), and subsequently, the oil phase is added beating 5 for minutes more. A milky liquid is obtained, and this sample is sonicated in Branson Sonifier 450 (Output control 10 and Duty Cycle 40%) in ice for 7 min in order to avoid the warming. The droplet size of the emulsions was measured after production in a DLS-Zeta-Sizer to assure the presence of monodisperse droplet distribution. The obtained droplet size was similar in all cases in order to eliminate effects from different surface area in the lipolysis (Torcello-Gómez, Maldonado-Valderrama, Martín-Rodríguez, & McClements, 2011).

2.3.3. Pancreatic lipase-catalyzed hydrolysis of emulsions

Determination of the pancreatic lipase activity by the pH-stat technique using olive oil from emulsion as substrate was carried out following the method described in (Torcello-Gómez et al., 2011). 3 mL emulsion were mixed with duodenal buffer to reach a final volume of 16 mL and bile salts (5 mg/ml). For this method, lipase (1.6 mg/ml lipase) is added to start the measurement. The inhibitors tested were incubated with lipase for 15 min before addition: Orlistat (0.44 mM), GSSG (1.4 mM) or silibinin(A) (1.8 mM). The free fatty acids released by the lipolysis of emulsions were titrated at a constant pH by sodium hydroxide (NaOH, 0.1 N) during the course of the hydrolysis. The titration is performed at 37 °C and at pH 7.0.

The experiment is based on measuring the volume of NaOH needed to maintain the pH constant at 7.0, for lipase exclusively, and in the presence of its inhibitors. The % of free fatty acids produced in each case was calculated by:

$$\%FFA = 100x\left(\frac{V_{NaOH}xm_{NaOH}xM_{lipid}}{W_{lipid}x2}\right) \tag{2}$$

where, V_{NaOH} is the volume of NaOH added, m_{NaOH} is the molarity of NaOH, M_{lipid} is the molecular weight of lipid used, and W_{lipid} is the weight of lipid used, assuming that lipolysis produces two FFA per one triacylglicerol molecule.

2.4. Analysis of in-vitro lipolysis in a single droplet

The interfacial tension assays were made following the methodology and protocols described in detail in Del Castillo-Santaella et al. (2015). Interfacial tension is measured in a pendant drop surface film described in detail elsewhere (Maldonado-Valderrama et al., 2015). The system is completely computer controlled with Software DINATEN® (UGR) and the detection and calculation of surface area and surface tension are automatic and based on Axisymmetric Drop Shape Analysis (ADSA) (Cabrerizo-Vilchez & a, Wege, H. a, Holgado-Terriza, J. a, & Neumann, a W. , 1999). The solution droplet is formed at the tip of a capillary which is immersed in a glass cuvette filled with the oil phase (Hellma®), hence simulating an oil–water emulsion and is kept in an externally-thermostated cell at 37C for all the experiments. Interfacial tension

was recorded at constant interfacial area (20 mm^2) and dilatational elasticity of the interfacial layer was measured at the end of adsorption curve at 5% amplitude and 0.1 Hz as described elsewhere (Del Castillo-Santaella, Sanmartín, Cabrerizo-Vílchez, Arboleya, & Maldonado-Valderrama, 2014, 2015). The adsorption curves and dilatational responses were recorded for lipase alone and in the presence of inhibitors as a rapid test of the inhibition of lipolysis induced by different compounds following the methodology described in detail in (Del Castillo-Santaella et al., 2015). The inhibitors tested were incubated with lipase for 15 min before addition at the same concentrations as in previous assays: Orlistat (0.44 mM), 1.4 M and silibinin(A) (1.8 mM).

2.5. Statistical analysis

Pancreatic lipase activity data represent mean values ± standard deviations unless otherwise indicated. To estimate the total inhibitory effect of the tested compounds, AUC were estimated. Statistical differences regarding the effect of Xenical, GSSG and silibinin(A) were estimated by a one-way ANOVA with a Tukey's *post hoc* test for multiple comparisons, using SPSS 25 software (SPSS Inc., Chicago, Illinois, USA). Concentration-effect data for agonists and antagonists were fitted by nonlinear regression, fitting the data to a variable slope model, using Prism 8.0 software (GraphPAD, CA, USA). Data were pooled from at least three different samples. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Search of potential inhibitors of pancreatic lipase active site

Pancreatic lipase is a target of great interest for the pharmaceutical industry and, in consequence, many previous inhibitors have been developed for obesity treatment. This situation and the availability of an X-ray structure of pancreatic lipase allowed us to apply docking based Virtual Screening for the search of compounds that interact in a similar way as other compounds whose inhibitory capacity has already been demonstrated (Egloff et al., 1995). After performing virtual screening calculations, top 100 compounds with highest docking score were retained for further visual analysis and to check, if they interacted with the lipase key residues. Next, commercial availability of compounds was checked by Molport®, and finally we selected and purchased Glutathione-disulphide (GSSG) and silibinin(A) as main hits (Fig. 2), with docking scores −12.2 and −12.8 kcal/mol, respectively. In comparison, orlistat and tributyrin yielded −11.1 and −7.62 kcal/mol.

Within the catalytic centre of pancreatic lipase, serine152 plays a pivotal role, since this residue forms a covalent bond with it with the *sn*-1 alkyl group necessary for the breakdown of the molecule (Egloff et al., 1995). Our results showed that orlistat, GSSG and silibinin(A) were able to strongly bind to this residue, suggesting their potential activity as lipase inhibitors (Fig. 2). Very close to serine152, a leucine residue (leu153) helps to stabilize the protein-drug interaction. Interestingly, our virtual screening analysis revealed that orlistat and silibinin(A) (but not GSSG) formed a hydrogen-bond with this residue. Other important difference regarding the interaction of GSSG and silibinin(A) with pancreatic lipase relies on the his263 residue (Fig. 2), other member of the catalytic triad of pancreatic lipase (Lowe, 1992). Our data showed that both orlistat, tributyrin and GSSG interact through salt bridges with the imidazole ring of his263 from lipase, while these salt bridges were absent in the case of silibinin(A) Finally, other important regulator of the pancreatic lipase activity, Phe77, which showed several interactions with all four compounds (Fig. 2), but while orlistat, tributyrin and silibinin(A) were strongly linked to Phe77 through an interaction mediated by two hydrogen-bonds, GSSG interaction was weaker (a detailed description of protein–ligand interactions is available as supplementary Table S1).

3.2. Inhibition of pancreatic lipase activity in solution

Firstly, basal lipase activity in solution was determined as a negative control of the inhibition of lipase, so then, we could compare with the maximum activity of this enzyme. In the same line, orlistat was used as positive control. On this way, we established how many units of lipase could be inhibited using orlistat (Table 1), which was considered as maximum inhibitory effect. Secondly, the activity of lipase was measured in the presence of inhibitors GSSG and silibinin(A).

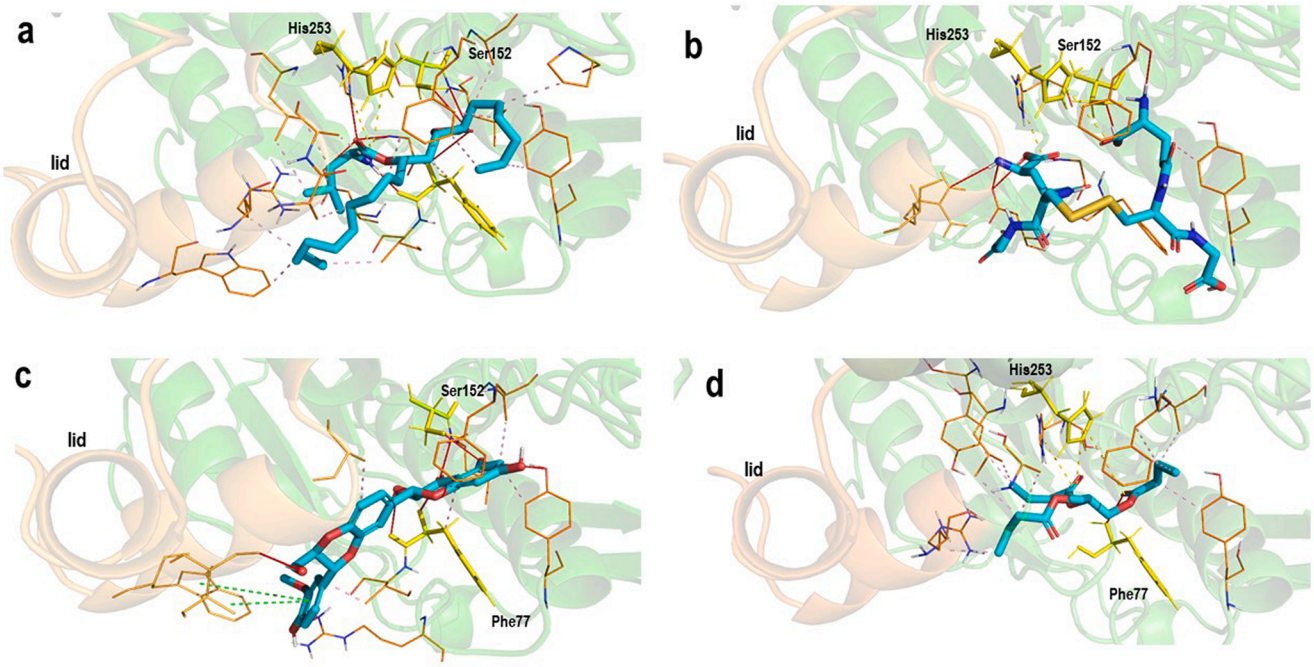

Fig. 2. 3D representation of molecular docking results of Orlistat (a), Glutathione-disulphide (b), Silibinin(A) (c) and Tributyrin (d) with pancreatic lipase (Protein Data Bank: 1lpb). Hydrogen bonds are shown in red, hydrophobic interactions in purple, salt bridges in yellow and pi-stacking interaction in green. Relevant residues within the catalytic centre are shown as yellow thin sticks. Other residues interacting with the compounds are shown as orange lines.

Table 1
Average enzymatic activity value of lipase at 1 mg dry weight per minute. The mean value was calculated using sections between 2 and 8 min of each assay, where the highest catalytic activity was performed. Values are obtained as a mean of at least three replicate measurements.

	Lipase	Lipase+Orlistat	Lipase+GSSG	Lipase+Silibinin(A)	*p* (ANOVA)
Enzymatic activity in dry weight U/(mg min)	30 ± 10	23 ± 10	15 ± 8*	24 ± 5	0.027

Data represent mean ± standard deviation. Statistical differences were analysed by a one-way ANOVA. *Post hoc Tukey's multiple comparisons test revealed significant differences especially between GSSG and Lipase activity ($p = 0.016$).

Surprisingly, results in Table 1 show that only GSSG displayed a significant inhibitory effect on lipase activity. Antioxidants are potential inhibitors of pancreatic lipase as demonstrated in literature studies (Li et al., 2018) and the reported inhibition is possibly associated with the antioxidant nature of GSSG (Surai, 2015). However, these authors use different methodologies to assess the inhibitory effects of lipase in solution. In fact, according to this experimental protocol to assess lipase activity, orlistat and silibinin provided similar values to lipase (Table 1). A reason for this is the different solubility in water of the inhibitor in the reaction site, which importantly affects the direct measurement of lipase activity. Orlistat and silibinin(A), are practically insoluble compounds in water, while GSSG is completely soluble in water. This different solubility could originate the different inhibitory activity found in solution. Orlistat is soluble in ethanol while silibinin (A) is soluble in acetone. The pancreatic lipase is inhibited clearly faster in the presence of GSSG owing to the high solubility of both compounds, which favours lipase inhibition, as measured in solution. However, this result is far away from the real situation during human digestion, where lipolysis occurs at oil–water interface of emulsified systems. Therefore, in order to obtain accurate and reliable information regarding lipolysis inhibition, it is important to develop more realistic models of lipase action, as it will be shown in the next section. Results shown in Table 1 will be just taken as fundamental information on the activity of compounds in solution, with no realistic applicability to the *in vivo* situation.

3.3. Inhibition of pancreatic lipase activity at oil–water interfaces

The inhibitory activity of silibinin(A) and GSSG on lipase action was evaluated using the rapid test based on interfacial tension measurements which was developed in a previous study (Del Castillo-Santaella et al., 2015). The experiment designed therein consisted in measuring the adsorption profile of Lipase + Inhibitor and compare it to the adsorption profile of lipase and Lipase + Orlistat as negative and positive controls, respectively. All the measurements were carried out in an aqueous drop immersed in the oil phase mimicking oil–water emulsion and therefore, the physiological conditions of the duodenum. The interfacial tension decrease (obtained after 1 h of adsorption) depends on the adsorption of lipase and on the lipolysis products at the interface, so that, the lower the interfacial tension, the higher lipolysis occurred. The positive and negative controls were previously identified (Del Castillo-Santaella et al., 2015) being negative control: 5 mN/m (no inhibition of lipase enzyme at oil–water interface) and positive control: 9 mN/m (full inhibition of lipase at the oil–water interface). Then, the dilatational elasticity of the adsorbed layer provides additional information on the molecular conformation, composition and mode of inhibition. However, the complexity of this magnitude does not allow establishing positive and negative compounds as with the interfacial tension (Del Castillo-Santaella et al., 2015).

With these premises, the interfacial tension and dilatational elasticity were measured for the potential inhibitors proposed in this study, silibinin(A) and GSSG and compared to lipase and Lipase + Orlistat at fixed concentrations. Final values after 1 h of adsorption are plotted in Fig. 3. Figure Supplementary materials 2 shows the dynamic adsorption curves measured to reach the final state of Lipase + Silibinin(A) and Lipase + GSSG at the oil–water interface compared with the adsorption profiles of lipase and Lipase + Orlistat given in Fig. 3.

On one hand, Fig. 3 shows no significant differences for Lipase + Silibinin(A) as compared to negative control, both in the interfacial tension and the dilatational interfacial elasticity. This result is possibly also related to the lack of solubility of silibinin (A) in water. Solutions were made in acetone, which is also poorly soluble in water and therefore this methodology does not seem to be valid for assessing the inhibitory potential of silibinin(A).

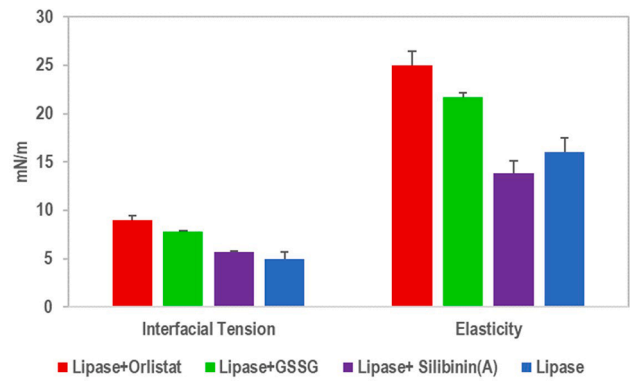

Fig. 3. Interfacial Tension and Interfacial dilatational elasticity obtained after 1 h of adsorption at constant interfacial area onto the olive oil water interface in duodenal buffer. Lipase (blue), Lipase + Orlistat (red), Lipase + GSSG (purple) and Lipase + Silibinin(A) (green). Concentrations: Lipase (1.6 g/l), Orlistat (0.22 g/l), GSSG (0.88 g/l) or Silibinin(A) (0.88 g/l). Values plotted are mean values of three independent measurements and standard deviations are plotted as error bars according to statistical tool. Then, mean values were compared by using the Tukey's test. Different letters were assigned at values with significant differences ($p < 0.05$).

On the other hand, both, the interfacial tension and dilatational interfacial elasticity of Lipase + GSSG do provide significant differences with respect to positive and negative controls (Fig. 3). The obtained value for the interfacial tension suggests that lipolysis is inhibited in the presence of GSSG, but to a lesser extent than the inhibition reached with orlistat. The higher value compared to negative control suggest the lower amount of lipolysis products at the interface. The interfacial dilatational elasticity also lies in between that of negative and positive controls providing further information on the intermolecular and intramolecular interactions occurring within the interfacial layer (Maldonado-Valderrama, 2019). A lower dilatational elasticity implies the formation of a less cohesive network of lipase molecules owing to the presence of lipolysis products at the interface. The dilatational elasticity obtained for Lipase + GSSG appears significantly higher than that of negative control (lipase) meaning again that there has been a significant inhibition of lipolysis in the presence of GSSG. Orlistat is covalently but reversibly bound to the serine residue of the active site of lipase, but it does not seem to disrupt the lipase molecule (Bénarouche, Point, Carrière, & Cavalier, 2014). Accordingly, the high dilatational elasticity of Lipase + Orlistat might be originated from the compact structure of lipase, as discussed in detail in Del Castillo-Santaella et al. (2015). The lower dilatational elasticity of Lipase + GSSG could indicate that the inhibiting mechanism of GSSG produces a conformational change of lipase which could also explain the slight loss of elasticity of the interfacial layer. This is consistent with virtual screening data, showing that GSSG digs down towards the catalytic centre, hence altering the

conformational structure of lipase.

3.4. Inhibition of in vitro lipolysis in a model emulsion

In view of previous results, the inhibition of pancreatic lipase by silibinin(A) and GSSG was further analysed in a more realistic model by measuring the release of FFA from an oil–water emulsion stabilized with Tween20 in the absence or presence of inhibitors. This methodology allows measuring *in vitro*, the lipolysis of model emulsions under conditions in the duodenum, being rather representative of the *in vivo* situation as recently shown (Deloid et al., 2018).

Fig. 4 shows the influence of different inhibitors on lipolysis in model olive oil emulsions as measured using the pH method described in Section 2.3.3. The rate and extent of FFA release decreased in the following order: Lipase > Lipase + GSSG > Lipase + Silibinin > Lipase + Orlistat (Fig. 4). The suppression of lipid digestion by silibinin(A) is therefore more significant than that of GSSG and importantly approaching that of orlistat. Regarding the solubility of silibinin(A), it seems to be improved by emulsification; the higher energy introduced in the system by agitation and sonication of the sample, the presence of emulsifier and bile salts and also the partial solubilisation in the oil phase could be responsible for the improved solubility reached. At any rate, results from Fig. 4 allow evaluating the inhibitory potential of silibinin(A) and also, confirm its high inhibitory potential.

Fig. 4 also quantifies the activity of lipase and Lipase + Inhibitor in olive oil emulsion. The presence of orlistat decreases in the activity of lipase by 71% and a similar inhibition was obtained with Lipase-Silibinin(A) (65%) in contrast to GSSG which shows a 46% inhibition of pancreatic lipase activity (Fig. 4). The inhibition can be further analysed by looking at the lag time and linear lipolysis rate (Chu et al., 2009). The lag time is defined as the time in minutes taken before lipolysis (Supplementary Table 2). This lag time followed the trend: lipase-orlistat > lipase-silibinin> lipase-GSSG> lipase. The maximum linear lipolysis rate can also be measured (g of consumed olive oil/min) obtaining lipase > lipase-silibinin(A) > lipase-orlistat > lipase-GSSG (Supplementary Table 2). The statistical studies showed similar rate for lipase-silibinin and lipase-orlistat and lipase-GSSG and lipase-orlistat (Supplementary Table 2). This agrees with results from Chu et al. who obtained similarly that the system which had less lipolytic activity presented a lag time 17.53 ± 0.51 min and maximum rate minor than the other systems (Chu et al., 2009).

These results allow extending and interpreting previous findings to more realistic conditions and also linking with structural aspects. Experimental results demonstrate that both inhibitors are promising agents in the development of natural compounds to reduce fat intake. However, the inhibitory effect of silibinin(A) seems more pronounced, than that of GSSG even approaching that of orlistat. The improved performance of silibinin(A) could be related to its improved interaction with hydrophobic sites in lipase which, seems to be a determinant factor in the inhibitory effects. It can be speculated that silibinin(A) inhibits pancreatic lipase activity mainly through the blockade of the conformational changes that expose the catalytic centre when lipase contacts the oil–water interface. Conversely, GSSG activity on pancreatic lipase seems to be more related to the occupation of the catalytic centre, causing a kind of hindrance for triglycerides turnover. Conformational changes upon adsorption at the oil–water interface proceed and the layer loses elasticity as discussed in the previous section.

This agrees with results from virtual screening. Silibinin(A) was able to interact with the amino acid trp252, which has been described as a crucial factor for the stabilization of the lid with the catalytic centre of pancreatic lipase. In addition, the virtual screening location of silibinin (A) suggests that it protrudes from the enzyme surface, so it could more easily contact the oil–water interface hence preventing lipolysis. He and co-workers have reported that the aromatic rings of polyphenol compounds interact hydrophobically with pancreatic lipase amino acids, phenylalanine and tyrosine (He, Lv, & Yao, 2006). This observation is in line with the predicted interactions between silibinin(A) and Phe77 and Trp252. Besides, another hydrophobic interaction was predicted between the hydroxyl groups of polyphenolic compounds and polar groups of pancreatic lipase (Martinez-Gonzalez et al., 2017). Both hydrophobic interactions will change the lipase conformational stability, resulting in a strong inhibitory activity.

Furthermore, Martinez-González and co-workers described that Quercetin, an effective lipase inhibitor, presented more polar interactions than others polyphenolic compounds on pancreatic lipase. This flavonoid has a high affinity towards proteins due to its structural characteristics. It is known that the number of rings and the free hydroxyl groups are associated with the ability of these compounds to interact with proteins (Martinez-Gonzalez et al., 2017). For these reasons, the polyphenols from different origins have been studied to inhibit

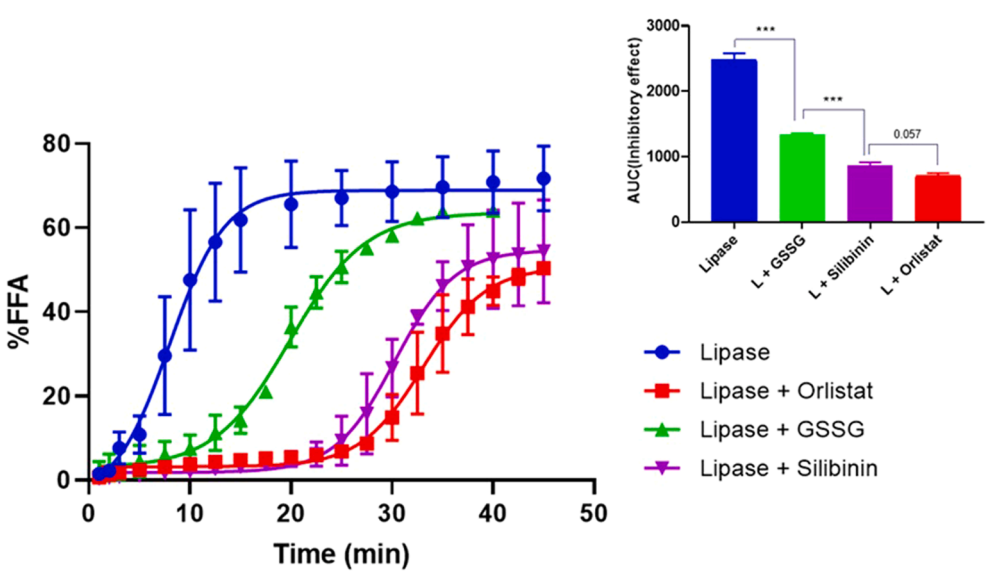

Fig. 4. The time dependence of the fatty acid release (%) from olive O/W emulsions stabilized with Tween 20, after adding lipase and bile salts (T = 37 °C). At least three measures were made. AUC of lipase, lipase-Orlistat, lipase-GSSG, lipase-Silibinin(A). Lipase (1.6 g/l), Orlistat (0.22 g/l), GSSG (0.88 g/l) or Silibinin(A) (0.88 g/l) were used at final concentrations.

the lipase in previous papers (Gondoin, Grussu, Stewart, & McDougall, 2010; Li et al., 2018; Vinodhini & Rajeswari, 2019). We find similarities between quercetin and silibinin(A) molecular structures, since the structure of quercetin is contained in site of silibinin(A) molecule (Fig. 1), which may explain the similar mechanism of action of quercetin and silibinin(A). It was also reported that Quercetin bound close to the active site of pancreatic lipase (Martinez-Gonzalez et al., 2017). The bind between lipase/Silibinin(A) would change the lipase structure, hence inhibiting its action. In the case of GSSG, the interfacial results correlate with the less inhibition capacity obtained as compared with orlistat. GSSG clearly inhibits lipase activity, preventing FFA release (Fig. 4). The presence of hydroxyl groups could interact with the active centre of pancreatic lipase. Furthermore, the antioxidant properties of this molecule could interfere and encourage the lipase inhibition.

According to the Virtual Screening, the main differences found in the interaction of the different inhibitors with lipase rely in the different interaction encountered with hydrophobic amino acids. The inhibition of lipase through hydrogen bounds in Gly76, Asp79, Arg256 and His151 for orlistat and GSSG were similar, although the interactions were hydrophobic in the case of silibinin (Ile78, Ala178 and Ala280) (Table Supplementary 2). There are some amino acids in common for both inhibitors like (Phe77 and Phe215) but the interactions are hydrogen bound for GSSG and hydrophobic for silibinin(A). Thus, orlistat, tributyrin and silibinin(A) bind to hydrophobic amino acid Leu, bind more strongly to Phe77 via hydrogen bonds (Fig. 2). Hence, these bindings might well reduce the hydrophobic sites in the complex, hence interfacial activity and ultimately lipolysis. Conversely, orlistat and GSSG bind to Lys, which is buried inside lipase, via salt bond (Fig. 2). This type of interaction possibly alters conformational stability of pancreatic lipase, hence reducing the elasticity of adsorbed lipase layer. Moreover, a conclusion from this study is that the lipolytic action is directly related to interaction with hydrophobic sites. Compounds that interact via hydrogen bonds, such as silibinin(A) and orlistat, reduce the hydrophobicity of lipase, providing an improved inhibition of lipolysis *in vitro*.

4. Conclusion

In summary, combination of *in silico* and *in vitro* studies of allow the rational investigation of new lipase inhibitors. The inhibitory potential is quantified for emulsified systems while interfacial and *in silico* studies allow further interpretation of the findings at the molecular level. The interaction with hydrophobic sites in lipase seems to be a crucial factor determining the lipolytic action of lipase. This is quantified by measuring the amount of free fatty acids released upon *in-vitro* lipolysis, being more efficient the inhibition when interaction with hydrophobic amino acids occurs. This is possibly related to the reduced interfacial activity of the complex when hydrophobic interaction occurs (silibinin (A) and orlistat) and with denaturation of lipase when interaction with polar amino acids buried in the molecule occur (GSSG). Results from this study confirmed that both GSSG and silibinin(A) are effective pancreatic lipase inhibitors, with potential as complements to dietary therapy for the treatment of obesity. Further *in vivo* and clinical studies are necessary to verify the effectiveness of these compounds for its use in humans, since, in addition to being natural compounds, their safety regarding human administration has already been demonstrated (Wah Kheong, Nik Mustapha, & Mahadeva, 2017).

Ethics statement

The present research did not include any human subjects and animal experiments

CRediT authorship contribution statement

Teresa Del Castillo-Santaella: Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Juan José Hernandez-Morante:** Conceptualization, Data curation, Investigation, Writing - original draft, Writing - review & editing. **Jesús Suarez-Olmos:** Data curation, Formal analysis, Methodology. **Julia Maldonado-Valderrama:** Data curation, Formal analysis, Methodology, Supervision, Resources, Writing - original draft, Writing - review & editing. **Jorge Peña-García:** Data curation, Formal analysis, Methodology. **Carlos Martínez-Cortes:** Data curation, Formal analysis, Methodology. **Horacio Perez-Sánchez:** Data curation, Formal analysis, Methodology, Supervision, Resources, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104479.

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