Identification of a new natural gastric lipase inhibitor from star anise

Jannet Kamoun^{1,2,3}, Renaud Rahier^{3,§}, Mohamed Sellami¹, Imed Koubaa⁴, Pascal Mansuelle⁵,
Régine Lebrun⁵, Alexandra Berlioz-Barbier³, Karine Alvarez⁶, Abdelkarim Abousalham³,
Frédéric Carrière², and Ahmed Aloulou^{1,§,*}

¹ University of Sfax, National School of Engineering of Sfax, Laboratory of Biochemistry and
Enzymatic Engineering of Lipases, Sfax 3038, Tunisia.

² Aix-Marseille University, CNRS, Bioénergétique et Ingénierie des Protéines, UMR 7281,
Marseille 13009, France.

³ Univ. Lyon, Université Lyon 1, Institut de Chimie et de Biochimie Moléculaires et
Supramoléculaires (ICBMS), UMR 5246 CNRS, Métabolisme, Enzymes et Mécanismes
Moléculaires (MEM²), Villeurbanne 69622, France.

⁴ University of Sfax, Faculty of Science of Sfax, Laboratory of Chemistry of Natural
Products, Sfax 3038, Tunisia.

⁵ Aix-Marseille University, CNRS, Institut de Microbiologie de la Méditerranée, Plateforme
Protéomique, Formation de Recherche 3479, Marseille 13009, France.

⁶ Aix-Marseille University, CNRS, Architecture et Fonction des Macromolécules
Biologiques, UMR 7257, Marseille 13009, France.

[§] Present Address: University of Illinois at Chicago, Department of Chemistry, Chicago,
Illinois 60607, USA.

* Corresponding author. Email: aloulou@uic.edu

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23 **Abbreviations**

24 DGL, dog gastric lipase; HPL, human pancreatic lipase; LipY, mycobacterial Rv3097c-
25 encoded lipase; SA, star anise; DMSO, dimethyl sulfoxide; M5ME, myricitrin-5-methyl ether;
26 FFA, Free fatty acid; TC4, tributyrin; NaTDC, sodium taurodeoxycholate; SAPRF, star anise
27 polyphenolic-rich fraction; MALDI-TOF-MS; matrix-assisted laser desorption ionization-
28 time of flight mass spectrometry; PMF, peptide mass fingerprinting; TLC, thin layer
29 chromatography; UPLC-HRMS, ultra-performance liquid chromatography high resolution
30 hybrid quadrupole-time of flight mass spectrometry.

31

32 **Abstract**

33 Bioactive compounds identification and isolation from natural complex samples, even though
34 being a difficult task, is of great interest in the drug discovery field. We describe here an
35 innovative strategy for the identification of a new gastric lipase inhibitor from star anise for
36 the treatment of obesity. After plant screening assays for gastric lipase inhibition, star anise
37 was selected and investigated by bioactivity guided fractionation. MALDI-TOF mass
38 spectrometry and peptide mass fingerprinting allowed the detection of an inhibitor covalently
39 bound to the catalytic serine of gastric lipase. Mass-directed screening approach using UPLC-
40 HRMS and accurate mass determination searching identified the flavonoid myricitrin-5-
41 methyl ether (M5ME) as a lipase inhibitor. The inhibitory activity was rationalized based on
42 molecular docking, showing that M5ME is susceptible to a nucleophilic attack by gastric
43 lipase. Overall, our data suggest that M5ME may be considered as a potential candidate for
44 future application as a lipase inhibitor for the treatment of obesity.

45 **Keywords**

46 Gastric lipase, inhibition; star anise, guided fractionation; mass spectrometry, molecular
47 docking.

1. Introduction

Gastric and pancreatic lipases play a key role in gastrointestinal digestion of dietary fat suggesting that they may represent a drug target for the treatment of obesity^{1,2}. Gastric lipase is the key enzyme for the gastrointestinal digestion of dietary triglycerides³. Lipid digestion is initiated in the stomach by the acid stable gastric lipase and continues in the duodenum through the synergistic actions of gastric lipase and colipase-dependent pancreatic lipase, leading to the formation of absorbable monoglycerides and free fatty acids (FFA)⁴. Inhibiting these lipases to reduce fat absorption is the main pharmacological approach adopted for the treatment of obesity⁵. Orlistat, a hydrogenated derivative of lipstatin derived from *Streptomyces toxitricini*, is the only FDA-approved anti-obesity drug that specifically targets digestive lipases⁶. This drug is an active site-directed inhibitor that reacts with the nucleophilic serine residue of the catalytic triad of digestive lipases within the gastrointestinal tract⁷. However, Orlistat, which is considered relatively too expensive to afford, is also known to inhibit various other enzymes and could lead to undesirable side effects, which might affect its current clinical use⁸. Therefore, looking for new cost-effective digestive lipase inhibitors is highly demanded nowadays.

Much interest has been shifted on plant polyphenols that have been reported to play a significant role in the prevention of overweight and obesity^{9, 10}. More precisely, polyphenolic-rich extracts from plant sources have been reported to inhibit pancreatic lipase *in vitro*¹¹⁻¹⁴.

Bioactivity guided fractionation is a useful approach for the chemical and biological screening of complex plant extracts^{15, 16}. Mass spectrometry (MS)-based affinity approaches have emerged as effective tools in small-molecule drug discovery. In these approaches the mass spectrometer can be exploited to rapidly screen drug candidates for specific interactions

with targets of interest ¹⁷. Coupling bioaffinity selection to MS has been developed in the screening of natural product extracts ¹⁸ and synthetic combinatorial libraries ¹⁹. Accordingly, direct and indirect bioaffinity screening approaches have been used. In the indirect screening approach, the complex protein-ligand is dissociated prior the MS analysis, allowing thus the identification of the target ligand. Whereas, in the direct screening approach, the MS analysis can be directly performed on the whole complex ²⁰. With the availability of MS technologies, direct bioaffinity screening approach could be a potential way to facilitate tentative identification of the relevant compound within simplified natural mixtures.

UPLC-HRMS is a modern powerful tool to identify known and unknown compounds in a complex mixture. The use of UPLC-HRMS in combination with a QTOF mass spectrometer for target and non-target screening purposes has gained popularity in recent years ²¹. Indeed, UPLC provides fast and high-resolution separation, which increases LC-MS sensitivity and minimizes matrix interference arising from minimal sample preparation. Coupling of UPLC system with QTOF-MS provides thus deep analysis on sample composition ²². Although increasing applications focus on analyzing molecules present in a variety of samples, there have been no reports regarding UPLC-HRMS application for identifying lipase inhibitors from natural product mixture.

To identify a natural gastric lipase inhibitor from plants, we proposed herein a new strategy based on bioactivity guided fractionation along with direct bioaffinity screening using MALDI-TOF-MS and peptide mass fingerprinting (PMF), non-target screening using UPLC-HRMS, and *in silico* molecular docking.

2. Materials and methods

2.1. Preparation of plant crude extracts for screening study

Dried plant species, purchased from local markets, were cleaned and finely powdered using a grinder. All powdered samples were separately extracted by maceration technique using hexane, ethyl acetate, ethanol and water (1:10 w/v ratio). The different extracts were evaporated to dryness *in vacuo* yielding dried extracts. All extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 100 mg/mL and were assayed for their anti-lipase potency.

2.2. Bioactivity guided fractionation

Star anise crude phenolic extract (SACPE) was prepared from star anise powder *via* maceration in ethanol:water (7:3 v/v) (1:10 w/v ratio) for 24 hours. The solid-liquid mixture was filtered with fritted glass number 3. Then, the filtrate was evaporated to dryness *in vacuo* yielding dried SACPE.

Star anise powder was extracted with hexane (1:10 w/v) for 48 h to remove lipoidal material ²³. After filtration, the filtrate was evaporated under vacuum to yield star anise hexanic extract. The residue was air-dried at room temperature overnight to get the dried defatted star anise (DFSA). Thus, star anise phenolic extract (SAPE) was prepared from DFSA *via* maceration in ethanol:water (7:3 v/v) (1:10 w/v ratio) for 24 h.

SAPE was successively fractionated using liquid-liquid partition and solid-liquid extraction to obtain star anise polyphenol-rich fractions (SAPRF). In a first step, liquid-liquid partitioning of SAPE with ethyl acetate gives two fractions SAPRF1 and SAPRF2. These fractions were evaporated under vacuum (45 °C), lyophilized and subjected to the inhibition test against DGL activity. SAPE, SAPRF1 and SAPRF2 were then analyzed by TLC on aluminum sheets coated with 0.2 mm silica gel 60 to characterize their chemical profile. The migration was performed with a mixture of chloroform:methanol (8:2 v/v). After that, the

spots were revealed with iodine vapor. In a second step, SAPRF2 was re-suspended in ethyl acetate for solid-liquid extraction. After 3 hours of maceration, the mixture was filtered. Similarly, this second step led to two fractions, SAPRF3 and SAPRF4, that were dried under vacuum. Subsequently, SAPRF2 and the fraction produced (SAPRF3 and SAPRF4) were subjected to DGL inhibition test and TLC analysis under the same experimental conditions of the first step. SAPRF4 was thereby selected for further investigations. A flow chart for the preparation of various fractions from star anise powder is given in **Fig. 1**.

2.3. Lipase activity measurements using the pH-stat technique

Recombinant DGL²⁴, HPL²⁵, and LipY²⁶ were produced and purified as described previously. Pancreatic colipase was purified from lipid-free porcine pancreatic powder²⁷. Lipases were stored in 150 mM NaCl buffered solutions (pH 6 for DGL, pH 7 for HPL, and pH 8 for LipY).

Enzymatic activity was assayed at 37 °C by measuring the amount of FFA released from a mechanically stirred tributyrin (TC4) emulsion, using 0.1 M NaOH with a pH-stat (Metrohm 718 STAT Titrino, Switzerland) adjusted to a fixed end point²⁸. TC4 emulsions were formed by mixing 0.5 mL TC4 with 14.5 mL buffer solution. The activities of DGL were determined using the following assay solution: 150 mM NaCl, 2 mM sodium taurodeoxycholate (NaTDC), and 2 mM bovine serum albumin at pH 5.5²⁴. In the case of HPL, the activities were determined using the standard assay solution for PL: 0.3 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl₂, and 4 mM NaTDC, in the presence of 5 molar excess of colipase²⁵. With LipY, the assay solution was 2.5 mM Tris-HCl (pH 7.5), 300 mM NaCl and 3 mM NaTDC²⁶. Under the above assay conditions, enzymatic activities were expressed as international units: 1 U = 1 μmol FFA released per minute. The specific activities of DGL, HPL, and LipY, expressed in U per mg of pure enzyme, were found to be 340 ± 21, 8021 ± 79 and 129 ± 2 U/mg, respectively.

2.4. Assay for anti-lipase activity

The lipase-inhibitor pre-incubation method was used to test, in aqueous medium and in the absence of substrate, the possible direct reactions between lipases and inhibitors⁵. Lipase-inhibitor pre-incubations were performed at 25 °C, at different times and at various inhibitor extract amounts (a_I), in presence of 4 mM NaTDC⁵. The final enzyme concentration in the incubation medium was 1 μ M. The residual lipase activity was measured by the pH-stat. The amount of the inhibitor fraction (a_{I50}) and the half-inactivation time ($t_{1/2}$), corresponding to 50 % of residual enzyme activity, were determined.

In each case, control experiments were performed in the absence of inhibitor fraction but with the same volume of DMSO. It is worth noting that DMSO at a final volume concentration less than 10 % has no effect on the enzyme activity.

2.5. UPLC-HRMS experiment

The system used for UPLC-HRMS was an ultra-performance liquid chromatography Ultimate 3000 (Thermo Fisher Scientific, Villebon-sur-Yvette, France) coupled to a high-resolution hybrid quadrupole-time of flight mass spectrometer (Impact II, Bruker, Brême, Germany) equipped with electrospray ionization source (ESI) (Bruker, Brême Germany). Instrument control and data collection were performed using Data Analysis 5.0 software.

Chromatographic separation was performed on a polar C18 column (10 cm $\times$ 2.1 mm $\times$ 1.7 μ m, Luna Omega, Phenomenex, France). The column oven temperature was set at 40 °C and an injection volume of 7 μ L of the sample was loaded. The sample compounds were separated at a flow rate of 0.4 μ L/min using (A) H₂O (Milli-Q) with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. The analytes were separated using the following gradient: from 0 % to 100 % of B in 30 min and kept constant for 5 min. The column was re-equilibrated for 10 min at the initial composition of the gradient before runs.

The ESI interface was operated on full scan mode (m/z 50-2000) in negative and positive ion mode. The parameters for the ESI ion source were as follows: the capillary voltage, 3.0 kV; the source temperature, 200 °C; the operating pressure of the nitrogen flow for the nebulizer gas, 45 Psi. Ultraviolet (UV) detectors measured the absorption over the range of 250 and 280 nm.

Before each acquisition batch, external calibration of high-resolution mass spectrometer was performed with a sodium formate cluster solution. The calibration solution was injected at the beginning of each run. MS/MS analyses were carried out with Data Dependent Acquisition mode for precursor ions with an intensity superior to 2000 counts using a stepping of collision energy ramp.

Annotation of each signal was performed by interrogation of different databases (ChemSpider, MassBank, Drugbank, HMDB) using a home-made software providing annotation of LC-MS data according to parent mass accuracy (< 5 ppm). After generation of a short list of potential candidates for each signal, the correlation of isotope patterns according to putative atomic compositions was checked to reduce the list of putative annotation ($mSigma < 30$). The investigation of experimental MS/MS data using web-based spectral database and/or *in silico* fragmentation tool as MetFrag© was necessary to obtain structural information and annotate more precisely each compound.

2.6. MALDI-TOF mass spectrometry analysis

DGL was pre-incubated for 30 min at 25 °C with a large excess of SAPRF4 ($a_I = 30 \mu g$) to abolish any residual lipolytic activity. A blank experiment was performed in the absence of SAPRF4. MALDI-TOF analysis of the entire non-inhibited or inhibited DGL was carried out with a Bruker Micoflex II mass spectrometer (Daltonik, Deutschland) using a saturated solution of α -cyano-4-hydroxycinnamic acid in acidified water (0.1 % TFA) and acetonitrile (30:70 v/v)²⁸. Mass spectra were acquired in the positive ion mode, using the Flex Analysis™

software program (Bruker, Daltonik, Deutschland). Protein identification was performed using the MASCOT™ version 2.2 search engine (Matrix Science, London, UK) and the NCBI protein database. Theoretical and experimental peptide mass were obtained using the BioTools™ software program (Bruker, Daltonik, Deutschland).

2.7. Peptide mass fingerprinting

Non-inhibited and inhibited DGL (with SAPRF4) were first separated by SDS-PAGE. The protein bands were then excised from the gel and subjected to in-gel trypsin digestion procedure as previously described²⁸. For peptides identification, the in-gel digested peptides were analyzed by a proteomic approach including MALDI-TOF/MS and electrospray ionization (ESI) quadrupole time of flight (QTOF) MS/MS (Waters, Manchester) coupled with a nano flow UPLC nano Acquity (Waters, Manchester). For UPLC-ESI-QTOF analysis, the samples were dissolved in the loading buffer (3% acetonitrile/ 0.1 % TFA in water) and desalted on a C18 nano trap (Symmetry C18, 180 µm × 2 cm, 5 µm, Waters) mounted on a 6-port valve, before on line elution onto a C18 column (BEH 130 C18, 100 µm × 10 cm, 1.7 µm, Waters). Gradient elution was performed from 3 % to 50 % of mobile phase B (100 % acetonitrile / 0.1 % formic acid) in A (0.1 % formic acid in water) for 30 min. The column was rinsed for 6 min with 85 % of B and then brought back in 1 min to the initial condition. Between each sample, a blank (injection of loading buffer only) was done using the same chromatographic method. The peptides were detected into the mass spectrometer in a positive ion mode using the MS^E mode. Data acquisitions of spectra were performed using the Micromass software Protein Lynx Global Server 2.5.2 (Waters).

2.8. Molecular docking

In silico molecular docking of the M5ME inhibitor present in the active site of DGL (PDB entry codes: 1K8Q - 2.70 Å resolution²⁴) was performed using AutoDock Vina²⁹ program under UCSF Chimera software³⁰. The grid box size was chosen to fit the whole

active site cleft and to allow non-constructive binding positions. The inhibitor structure model was built using the Avogadro program³¹. Binding modes were scored and ranked based on the most favorable energies.

3. Results

3.1. Screening for anti-lipase properties of crude medicinal plant extracts

Twenty extracts, prepared from five medicinal plants with anti-obesity potential (*Illicium verum*, *Glycyrrhiza glabra*, *Salvia officinalis*, *Thymus vulgaris*, and *Rosmarinus officinalis*)³², were screened for their inhibitory effect against DGL activity at an a_i value of 1 mg. The ethanolic extract of the fruits of *Illicium verum* (star anise) was found to have the strongest inhibitory activity (81 %) against DGL (**Table S1**). While the ethanolic extract of *Rosmarinus officinalis* and the roots of *Glycyrrhiza glabra* showed a moderate inhibitory activity (43 and 44 %, respectively) toward DGL, the remaining medicinal plant extracts displayed weak inhibitory activities. Star anise was thereby selected for further processing.

3.2. Effect of defatting star anise on anti-lipase activity potency

Since star anise hexanic extract showed a weak inhibitory rate (18 %, **Table S1**) and a marked yield (around 7 %, data not shown), we evaluated the anti-lipase activity of phenolic extract from star anise before and after defatting sample. Thus, SACPE (non-defatted) and SAPE (defatted) extracts were prepared as described in the experimental section. Using an a_i value of 1 mg of each extract, the lipase inhibitory activity of SAPE (100 %) was higher than that of SACPE (89 %).

3.3. Bioactivity guided fractionation of SAPE

SAPE was fractionated using the scheme shown in **Fig. 1**. Two steps were used to fractionate SAPE. In the first step, the fractionation of SAPE led to SAPRF1 and SAPRF2, which were analyzed on TLC (**Fig. S1A**). One can note that spots intensities in SAPRF1 and

SAPRF2 have increased compared to the initial extract (SAPE). DGL activity inhibitory test was conducted on each of these two fractions. At an a_1 value of 500 μg of each fraction, SAPRF2 totally abolished DGL residual activity, while SAPRF1 led only to 25 % residual DGL activity. Subsequently, as SAPRF2 fraction was found to be more active, it was further subjected to a second fractionation step. Similarly, this latter led to SAPRF3 and SAPRF4 (**Fig. 1**) which were subjected to lipase activity inhibitory test and TLC analysis (**Fig. S1B**). Unlike SAPRF3, SAPRF4 was found to display promising anti-lipase potency with 100 % DGL inhibition rate at an a_1 value of 250 μg . Regarding TLC analysis, obtained results showed that SAPRF3 is moderately polar fraction, while SAPRF4 seems to have a strong polar character.

3.4. Evaluation of the inhibitory effect of SAPRF4 against lipases of medicinal interest

The inhibitory activity of the SAPRF4 fraction was evaluated against two mammalian digestive lipases (DGL and HPL) and one microbial lipase belonging to the hormone-sensitive lipase (HSL) family (LipY)²⁶. With each lipase, linear kinetics corresponding to the FFAs (μmol) released *vs.* time (min) were obtained in the presence and absence of the inhibitor fraction. A dose-dependent effect was observed upon increasing the amount of SAPRF4 on DGL, HPL and LipY (**Fig. 2**). At an a_1 value of only 30 μg , DGL activity was totally abolished after a 30 min incubation period (**Fig. 2A**), while HPL was strongly inactivated (96 %) at an a_1 value of up to 1 mg (**Fig. 2B**). However, LipY activity was not completely abolished even at a high SAPRF4 amount, showing a residual activity of ~20 % (**Fig. 2C**). The a_{150} values were found to be 7 μg , 82 μg and 5 μg for DGL, HPL and LipY, respectively (**Table 1**), indicating that SAPRF4 is a potent inhibitor toward both DGL and LipY.

The influence of the incubation time on the level of inhibition of DGL, HPL and LipY by the SAPRF4 fraction was further investigated (**Fig. 2D-F**). The residual activity of these

lipases decreased rapidly and reached a plateau value after approximately 30 min of incubation. From these inhibition curves, values of the half-inactivation times ($t_{1/2}$) were then determined and found to be 0.4 min, 0.6 min and 0.1 min for DGL, HPL and LipY, respectively (**Table 1**). Such values reflect an extremely high rate of inhibition of these lipases by SAPRF4.

3.5. Analysis of the inhibitor-modified DGL complex with mass spectrometry

To investigate whether the inhibitory compound within SAPRF4 forms a covalent bond with the catalytic serine of DGL, MALDI-TOF-MS analysis was conducted on both non-inhibited and inhibited DGL. **Fig. 3** shows typical spectral recordings of untreated and treated DGL with SAPRF4. A clear shift in the molecular mass of DGL was observed reflecting a covalent binding of the inhibitor to the lipase.

To deduce the exact molecular mass of the inhibitor contained in the SAPRF4 fraction, PMF analysis of non-inhibited and inhibited DGL was performed. Results showed that the peptide L¹⁴⁷-K¹⁶⁸ (LHYVGHSQGTIGFIAFSTNPK) containing the catalytic S¹⁵³ residue was detected at a molecular mass of 2376.05 Da with non-inhibited DGL (**Fig. 4A**). This catalytic peptide appeared concomitantly with a second peptide detected at a molar mass of around 2414.82 Da. By contrast, when DGL was incubated with SAPRF4, a mass increase of + 489.52 Da was observed in the PMF spectrum for the catalytic peptide (**Fig. 4B**), while the peptide detected at a molar mass of around 2414.82 Da remained unchanged. This mass shift (489.52 Da) observed here is supposed to correspond to the molecular mass of the natural inhibitor bound to DGL.

To tentatively identify the molecular formula and the structure of the phytochemical compound that binds covalently to DGL, we analyzed the SAPRF4 fraction by UPLC-HRMS. Seven major phenolic compounds have been identified (**Fig. S2A and Table S2**). To empirically calculate their potential molecular formulas, various criteria have been used

among them: the isotopic pattern, the fragmentation pattern and the high-resolution accurate mass (with error below 0.4 ppm at the time of matching the theoretical mass with the measured mass). Mass-directed identification showed that the natural DGL inhibitor compound within the SAPRF4 fraction might correspond to myricitrin-5-methyl ether (M5ME) with a molecular mass of 479.1184 Da and a molecular formula of $C_{22}H_{22}O_{12}$ (**Fig. 5**).

3.6. Molecular docking

To shed light on DGL-M5ME binding interactions, *in silico* molecular docking was performed using AutoDock Vina program. The open conformation of DGL, in complex with a phosphonate inhibitor covalently bound to its catalytic serine residue ²⁴, was used as reference model to improve the reliability of docking results. The lipase active site was found to be fully accessible thus enabling docking experiments. Automated docking resulted in several possible conformations of the M5ME inhibitor within DGL catalytic pocket, with favorable binding energies ranging from -8.3 to -9.5 kcal/mol (**Fig. S3A**). For subsequent analysis, we retained the best matching conformation that directly exposes the most reactive carbon atom of the M5ME inhibitor towards the catalytic serine, within a calculated distance of 3.8 Å which is sufficient to promote the nucleophilic attack (**Fig. S3B**). In this configuration, we found that M5ME could fit properly into DGL catalytic pocket (**Fig. 6**), which consists of a deep canyon of approximately 20 Å long, 7 Å wide, and 20 Å deep ²⁴. Moreover, like the phosphonate inhibitor, M5ME shows a relatively flat structure, with measured lengths of around 12 Å long, 5.5 Å wide, and 9 Å deep (**Fig. S3C**). For the sake of comparison, the previously reported phosphonate inhibitor only measures 6 Å long, 2 Å wide and 10 Å deep, and thus requires the presence of β-octyl glucoside or 1-amino-anthracene to completely fill the catalytic pocket ²⁴. Interestingly, a more detailed analysis of the environment of the M5ME compound, bound to the catalytic serine, revealed a significant stabilizing effect of the DGL-M5ME tetrahedral

intermediate by the neighboring oxyanion hole (**Fig. 5**). Indeed, an oxygen originating from the pyran ring of M5ME points towards the backbone nitrogen atoms of Leu67 and Gln154 residues within hydrogen bond distances of 2.37 Å and 2.08 Å, respectively.

4. Discussion

Star anise (*Illicium verum*) is an important traditional Chinese medicine as well as a commonly used spice. Its tree is an aromatic evergreen tree growing almost exclusively in southern China and Vietnam. The fruit of star anise is used in traditional medicine to treat stomachache, colic, vomiting, insomnia, skin inflammation, rheumatic pain, dyspepsia, facial paralysis, asthma, and bronchitis³³. A great deal of research effort is being devoted to testing the putative beneficial effects of star anise extracts. Modern pharmacology studies demonstrated that crude extracts and active compounds of star anise possess wide pharmacological actions³⁴. However, to the best of our knowledge, none has yet examined the effect of star anise extracts on digestive lipases activities.

DGL provides a good model for human gastric lipases^{24, 35}. A screening of a collection of 20 extracts from five medicinal plants using solvents with different polarities allowed the identification of ethanolic extract from star anise, which efficiently inhibited gastric lipase (**Table S1**). The inhibitory effect of star anise ethanolic extract on DGL might be caused by the presence of phenolic compounds within the extract. It was reported that ethanol/water extracts of star anise contained high amounts of phenolic compounds³⁶. The use of water in combination with ethanol contributes to the creation of a moderately polar medium that ensures the extraction of phenolic compounds³⁷. Plant phenolic compounds, such as flavones, flavanols, tannins and chalcones, have been reported as pancreatic lipase inhibitors¹⁴, thus supporting the use of star anise polyphenolic-rich extract for further bioactivity guided fractionation (**Fig. 1**).

Our results showed that removal of lipoidal material from star anise before phenolic extraction enhanced anti-lipase activity, which might be related to the fact that the concentration of phenolics in SACPE is lower than in SAPE, suggesting thereby that the anti-lipase effect is related to star anise phenolic compounds. One of the hallmarks of bioactivity guided fractionation is the trend of increasing potency with increasing fractionation. Indeed, the a_I value that totally abolishes gastric lipase activity moved from 1 mg SAPE to 30 μ g SAPRF4 after two fractionation steps. According to TLC analysis, SAPRF4 produced by this fractionation approach is a less complex mixture than the initial SAPE (**Fig. S1**). We therefore sought to evaluate SAPRF4 potency and selectivity against other lipases of medicinal interest.

Inhibiting lipases has potential applications in the field of medicine. The digestive lipase inhibitor Orlistat used for the treatment of obesity has been shown to also inhibit HSL³⁸ and microbial lipases^{8, 39}. In the sake of comparison, SAPRF4 was found to inhibit efficiently DGL, HPL, and LipY (a microbial lipase belonging to the HSL family). The ability of SAPRF4 to inhibit the catalytic activity of these lipases was assessed in terms of a_{I50} and $t_{1/2}$ values (**Table 1**). The inhibition of HSL could be a potential approach to reduce levels of circulating FFAs linked to insulin resistance in obese patients⁴⁰. Consequently, SAPRF4 might be an effective candidate for the treatment of obesity and diabetes. Moreover, as LipY contributes to the growth and pathogenicity of *M. tuberculosis*²⁶, it would be a therapeutic target for SAPRF4 as an anti-microbial agent.

On the bioaffinity interaction level, inhibitors can bind either covalently or non-covalently with their biological target⁴¹. In the cases of non-covalent inhibition, the inhibitor does not bind covalently with the biological target. Thereby, non-covalent inhibitor compounds are of no special interest in drug discovery projects¹⁶. MALDI-TOF mass spectrometry is an analytical instrument tool that can measure protein-inhibitor complex mass when a specific covalent binding occurs, reflecting thereby an approximate molecular mass of

the inhibitor³⁸. Direct MALDI-TOF mass spectrometry analysis of DGL totally inhibited by SAPRF4 showed the occurrence of an increase in the molecular masses of DGL, which is compatible with the formation of a covalent complex with the inhibitor. PMF analysis of a trypsin digest of DGL inhibited or not with SAPRF4 showed an increase in the molecular mass of the catalytic serine-containing peptide (**Fig. 4**) corresponding in size to the approximate molecular mass of the inhibitor. This process might involve a nucleophilic attack between the enzyme's catalytic serine and the inhibitor, leading to the formation of a covalent inhibitor-modified enzyme. Based on PMF analysis, it has been also shown previously that synthetic inhibitors, oxadiazolones²⁸ and cyclophostin analogs^{8, 42}, form a covalent bond with the catalytic serine residue of lipases. To the best of our knowledge, this is the first study that used covalent inhibitor-modified enzyme complex data to tentatively identify a lipase inhibitor in a natural extract.

Achieving the approximate molecular mass of the covalent lipase inhibitor, the identification of the compound of interest within SAPRF4 would be rather more straightforward. Thus, using the inhibitor mass information, we applied UPLC-HRMS for mass-directed screening to tentatively identify the expected lipase inhibitor in SAPRF4. Using this non-target screening, it was possible to separate and detect seven chromatographic peaks containing high resolution MS information (**Fig. S2**). The tentative identification of these peaks was performed according to their high-resolution accurate mass and isotopic patterns to empirically determine their molecular formulas. By inspecting all mass spectra around m/z of 489.52 in the chromatogram of SAPRF4, one ion having m/z of 479.1187 was found to fit with PMF analysis data. This ion, assigned to the molecular formula $C_{22}H_{22}O_{12}$, corresponds likely to the flavonoid myricitrin-5-methyl ether (**Fig. 5**) that has not been identified previously from star anise.

To gain insights into the lipase-inhibitor binding interactions at the molecular level, M5ME was docked into the active site of DGL (**Fig. 6**). The final selection of M5ME “bioactive” conformation suggests that the inhibition mechanism involve a nucleophilic attack by the hydroxy group of the catalytic Ser on a reactive carbon atom of the inhibitor pyran ring, thus leading to the formation of a stoichiometric enzyme-inhibitor covalent complex (**Fig. 5**). Interestingly, the nucleophilic attack could be favored by some surrounding residues, including the backbone nitrogen atoms of Leu⁶⁷ and Gln¹⁵⁴ that belong to the previously described DGL oxyanion hole ²⁴.

Much more interest has been shifted on plant flavonoids for their possible anti-obesity effects. *In vitro* and *in vivo* studies provided evidence on the potential role that flavonoids play in the management of obesity ⁴³. M5ME, a myricetin glycoside, was isolated from the flower of *Rhododendron poukhanense* as a potent antioxidant flavonoid. Although no lipase inhibiting activity was reported in the literature for M5ME, the structurally fairly similar quercetin and kaemferol were reported to be potent pancreatic lipase inhibitors ⁴⁴. Moreover, myricetin was found to exert *in vivo* a strong anti-obesity and anti-hyperlipidaemic activities by modulating the lipid metabolism ⁴⁵.

To our knowledge, this is the first report that tentatively identified a new natural gastric lipase inhibitor from a natural product. The combination of *in silico* molecular docking results with those of lipase inhibition and mass spectrometry provides a scientific evidence for the potential medicinal use of the flavonoid myricitrin-5-methyl ether as a lipase inhibitor for the treatment of obesity, type 2 diabetes and tuberculosis.

Author contributions

J.K. carried out experiments and performed analyses. R.R., M.S., P.M., R.L. and A.B.-B. contributed to experiments realization and analysis. I.K., K.A., A.A. and F.C. contributed to experiments design and analysis. J.K. and A.A. conceived experiments and wrote the paper.

Declaration of interests

There are no conflicts of interest to declare.

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564

565 FIGURE LEGENDS

566 **Fig. 1. Bioactivity-guided fractionation scheme of star anise.**

567

Fig. 2. Evaluation of the inhibitory effect of the SAPRF4 fraction against DGL, HPL and LipY lipases. Each lipase was pre-incubated in the presence of various SAPRF4 amounts for 30 min at 25 °C and residual activities of DGL (A), HPL (B) and LipY (C) were measured. Alternatively, residual activities of DGL (D), HPL (E) and LipY (F) were measured as a function of the incubation time at a constant SAPRF4 amount. The a_I value used in these latter experiments was chosen so that the average residual activities of DGL, HPL and LipY obtained after 30 min of incubation were in the 15-20 % range. Results are expressed as mean values of at least three independent assays.

577 **Fig. 3. MALDI-TOF mass spectrometry analysis of untreated (grey spectrum) and**
578 **treated (black spectrum) DGL. A 30-min incubation was performed at 25 °C in the**
579 **presence or absence of SAPRF4 amount of 30 µg.**

580

Fig. 4. PMF spectra of untreated (A) and treated (B) DGL. A 30-min incubation was performed at 25 °C in the presence or absence of SAPRF4 amount of 30 µg. Left panels, region of the unmodified isotopic peptide L¹⁴⁷-K¹⁶⁸ containing the catalytic Ser¹⁵³ and detected at 2376.05 Da. This peak is overlaid with a second one corresponding to the peptide detected at 2414.82 Da. Right panels, region in which a new isotopic peptide was expected to occur, resulting from the covalent binding of the natural inhibitor of interest to the catalytic serine. Mass shift was calculated as the difference between the experimental m/z of the unmodified peptide and the theoretical m/z of the unmodified peptide.

Fig. 5. Chemical structure of the flavonoid myricitrin-5-methyl ether (M5ME) and proposed mechanism underlying DGL inhibition by M5ME. The nucleophilic attack by the hydroxyl group of the catalytic Ser¹⁵³ residue on the inhibitor reactive site leads to a 1,4-nucleophilic addition on a double bond conjugated to a ketone and the formation of an enol function.

Fig. 6. Visualization of DGL-M5ME binding interaction by molecular docking. A, molecular surface representation of DGL active site crevice with the bound M5ME. B, view of the active site of DGL with the bound M5ME. The backbone nitrogens of residues Leu⁶⁷ and Gln¹⁵⁴ are at hydrogen bonding distance from an oxygen atom originating from the inhibitor. The catalytic residues Ser¹⁵³, His³⁵³ and Asp³²⁴ are highlighted in pink and the inhibitor is represented in CPK coloring (oxygen, red).

604 **Table 1.** a_{I50} and $t_{1/2}$ values of SAPRF4 on DGL, HPL and LipY.^{*}

605

Lipases	a_{I50} (μg)	$t_{1/2}$ (min)
DGL	7	0.4
HPL	82	0.6
LipY	5	0.1

606

607 ^{*} SAPRF4 amount (a_I) used for the determination of $t_{1/2}$ was 10 μg, 200 μg and 10 μg for
608 DGL, HPL and LipY, respectively. Results are expressed as mean values of at least three
609 independent assays (CV % < 5.0%).

610