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Your Ref: (Please advice)
Our ref: SK/P1515/UPM/16 (Please use our application number in all future correspondence)

July 25, 2016

Prof. Raja Noor Zaliha Raja Abd Rahman
Enzyme and Microbial Technology
Research Centre
Faculty of Biotechnology & Biomolecular Sciences
Universiti Putra Malaysia (UPM)
43400 Serdang Selangor Malaysia

Post (Confirmation copy)

Re: Malaysian Patent Application No: PI2016702034
Date of Filing: 02th June 2016
Applicant: UNIVERSITI PUTRA MALAYSIA
Invention: **ROSMARINIC ACID AS PANCREACTIC LIPASE INHIBITOR** (Application no: PI2016702034)

We have the pleasure in confirming that we have filed the above identified Patent application at the Intellectual Property Corporation of Malaysia on 02th June 2016. Herewith, we enclose the filed copy of Form 1. A Original copy of the Certificate of Filing will be forwarded upon receiving it from MyIPO and receiving the additional payment.

Now that you have filed a patent application in Malaysia, should you require to file a foreign patent application for the same invention, you may do so by filing a foreign application before **02 June 2017.** The above deadline is in accordance with the PARIS Convention or Patent Cooperation Treaty (PCT) if priority is to be claimed. Please note that, this date is non extendable. We would be able to assist you in filing of patent application in any country.

Please note that; the above application(s) will be disclosed to public by the Malaysian IP office at the 18th month from the date of filing the application. Should you wish to withdraw to disclose your application you may do so by giving us an advance notice within 2 weeks of the scheduled publication date. Please take note that we shall endeavour but accept no responsibility in reminding you on the dates filing and request for substantive examination. Therefore, we would advise you to make note on the dates filing and request for substantive examination. Please also keep us duly informed of any change of address.

Thank you for entrusting us with the application. We have enclosed our debit note (**Invoice 1873**) for full payment (including service tax, request for Substantive examination). We have enclosed a copy of Form 17, which is to be duly signed and return to us as soon as possible by 03 August, 2016.

Yours sincerely,

Sushil Kaur
IP Director
AETAS IP Solutions Sdn.Bhd.

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CERTIFICATE OF FILING

APPLICANT	:	UNIVERSITI PUTRA MALAYSIA (UPM)
APPLICATION NO	:	PI 2016702034
REQUEST RECEIVED ON	:	02 JUNE 2016
FILING DATE	:	02 JUNE 2016
AGENT'S/APPLICANT'S FILE REF.	:	SK/P1515/UPM/16

Please find attached, a copy of the Request Form relating to the above application, with the filing date and application number marked thereon in accordance with Regulation 25(1).

Date : 21 JULY 2016

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Patents Form No.1 PATENTS ACT 1983 REQUEST FOR GRANT OF PATENT (Regulations 7(1)) To: The Registrar of Patents Patents Registration Office Kuala Lumpur, Malaysia	For Official Use APPLICATION NO: **PI 2016702034** Filing Date: **02/06/2016** Fee received on: **02/06/2016** Amount: **RM570**
Please submit this Form in duplicate together with the prescribed fee	Applicant's file reference: **SK/P1515/UPM/16**

THE APPLICANT(S) REQUEST(S) THE GRANT OF A PATENT IN RESPECT OF THE FOLLOWING PARTICULARS:

I. TITLE OF INVENTION: **ROSMARINIC ACID AS PANCREACTIC LIPASE INHIBITOR**

II. APPLICANT(S) (the data concerning each applicant must appear in this box or , if the space insufficient, in the space below):

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Additional Infomation (if any)

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A statement justifying the applicant's to the patent accompanies this Form
Yes [√] **No**

Additional Information (if any)

IV.AGENT OR REPRESENTATIVE:

Applicant has appointed a patent agent in accompanying Form No. 17 Yes [√] No
Agent Registration No: **PA/2008/0192**
Applicant has appointed to be their representative: **SUSHIL KAUR A/P GURNAM SINGH**

V. DIVISIONAL APPLICATION:

This application is a divisional application
The benefit of the filing date priority date
of the initial application is claimed in as much as the subject-matter of the present application is contained in the initial application identified below :
Initial Application No:
Date of filing of initial application:

Additional Information (if any)

VI. DISCLOSURE TO BE REGARDED FOR PRIOR ART PURPOSES:

(a) Disclosure was due to acts of applicant or his predecessor in title

Date of disclosure:

(b) Disclose was due to abuse of rights of applicant or his predeceesor in title

Date of disclosure:

A statement specifying in more detail the facts concerning the disclosure accompanies this Form. Yes No √

Additional Information (if any)

VII. PRIORITY CLAIM (if any)

The priority of an earlier application is claimed as follows :
Country (if the earlier application is a regional or international application, indicate the office with which it is field) :
Filing Date:
Application No:
Symbol of the International Patent Classification:
If not yet allocated, please tick
The priority of more than one earlier application is claimed

Yes No √

The certified copy of the earlier application(s) accompanies this Form

Yes No √

If No, it will furnished by Date:

Additional Information (if any)

VIII.CHECK LIST

A. This application contains the following:

1. Request	1	sheets
2. Description	11	sheets
3. Claim	3	sheets
4. Abstract	1	sheets
5. Drawings	3	sheets
Total	19	sheets

B. This Form, asfiled, is accompanied by the items checked below :

(a) Signed Form No. 17
(b) Declaration that inventor does not wish to be named in the patent
(c) Statement justifying applicant's right to the patent
(d) Statement that certain disclosure be disregarded
(e) Priority document (certified copy of earlier application)
(f) Cash, cheque, money order, bank draft or postal order for the payment of application fee
(g) Other documents (specify)

IX.SIGNATURE: **mail=sushil@aetas.com.my, cn=SUSHIL KAUR, ou=Contact Number - 0162121095,ou=Identity Card / Passport No -###############, ou=Terms of use at www.msctrustgate.com/rpa (c)00, ou=Bahagian Teknologi Maklumat V2, o=Perbadanan Harta Intelek Malaysia, l="D6-SunwayPJ@51A, Jalan SS9A/19,(Off Federal Highway),Section 51A,", st="47300, Petaling Jaya,Selangor Darul Ehsan.", c=MY, CertSerialNo=3cbfda88e753af3eddd38ee77bfea47a|**

02/06/2016 (Date)

**(~~Applicant~~/Agent)

If Agent, indicate Agent's Registration No.: **PA/2008/0192**

For Official Use
Date application received: **02/06/2016**
Date of receipt of correction, later filed papers or drawings completing the application: **-**

* Delete whichever do not apply
** Type name under signature and delete whichever do not apply

ROSMARINIC ACID AS PANCREACTIC LIPASE INHIBITOR

Field of the Invention

The present invention pertains to rosmarinic acid for use in the inhibition of pancreatic lipase. The use of the present invention may be applied in medical and dietary methods for the reduction of body fat in mammals. The invention is in particular useful for the treatment of metabolic disorders such as obesity or diabetes.

Background of the Invention

Obesity is defined as an excessive fat accumulation detrimental to human health. It has also been defined as increased adipose tissue mass as a result of an enlargement in fat cells or an increase in their number (Couillard et al., 2000). A crude measure of obesity is the Body Mass Index (BMI), calculated as body weight in kilogram divided by the square of height in meters. Overweight is defined as a BMI of 25.0–29.9 kg/m2, and a BMI exceeding 30 kg/m2 is considered as obese. Extreme obesity is defined as a BMI of greater than 40 kg/m2 (Thompson et al., 2007). However, there is a demand for a more limited range for BMI classification in Asia because of the high prevalence of comorbidities, particularly diabetes and hypertension (James et al., 2001). Another approach to assessing obesity is measuring waist to hip ratio (WHR), which is a better indicator of other severe health conditions. The National Institute of Diabetes, Digestive, and Kidney Diseases, USA, has indicated that men with waist–hip ratios of more than 1.0 and women with more than 0.8 are at greater health risk as a consequence of their fat distribution.

Lipases are enzymes that digest fats, including triacylglycerol and phospholipids. Human lipases include the pre-duodenal (lingual and gastric) and the extra-duodenal (pancreatic, hepatic, LPL and the endothelial) lipases (Mukherjee, 2003). Pancreatic lipase (triacylglycerol acyl hydrolase), the principal lipolytic enzyme, plays a key role in the efficient digestion of triglycerides. It removes fatty acids from the α and α' position of dietary triglycerides, yielding β-monoglycerides and long chain saturated and polyunsaturated fatty acids as lipolytic products (Mukherjee, 2003; Shi and Burn, 2004; Thomson et al., 1997). Pancreatic lipase, encoded by the PNLIP gene in humans is secreted into the duodenum through the duct system of the pancreas. Pancreatic lipase is responsible

for the hydrolysis of 50–70% of total dietary fats (Birari and Bhutani, 2007). The primary structure of the pancreatic lipase was determined by analysis of recombinant cDNA clones derived from a cDNA library of human pancreas. The sequence was found to be a single chain glycoprotein of 449 amino acids while the three-dimensional structure of human pancreatic lipase was solved by X-ray crystallography. The encoded protein shows 86% and 68% homology with porcine and canine pancreatic lipase, respectively (Thomson et al., 1997).

Pancreatic lipase inhibition is one of the well-known modes of action for the determination of the potential efficacy of natural products as antiobesity agents (Sugiyama et al., 2007). Currently, the only lipase inhibitor available in the market which has obtained approval by the FDA for obesity treatment is tetrahydrolipstatin, a chemically-derived analogue of lipstatin (Weibel et al., 1987) from Hoffmann La Roche (Basel, Switzerland). It is commonly known as orlistat and is marketed as Xenical® (Manufacturer: Roche) or alli® (Manufacturer: GlaxoSmithKline). In Malaysia, orlistat is accessible by a brand name Cuvarlix®, which is marketed by Pharmaniaga (Figure 2.1). Orlistat was derived from lipstatin which was isolated from the mycelium of Streptomyces toxytricini (a soil microbe) in 1987 by Weibel et al. Orlistat inhibits pancreatic lipase in an irreversible manner with an IC50 of 0.14 μM. Orlistat contains a β-lactone moiety that is essential for activity (Weibel et al., 1987). Orlistat is the example of successful pancreatic lipase inhibitor, up to now. However, it has several gastrointestinal side effects such as oily stools, oily spotting, and flatulence (Birari and Bhutani, 2007). Due to these reasons, many researchers have attempted to obtain newer pancreatic lipase inhibitors that lack these unpleasant side effects.

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is commonly found in species of *Boraginaceae* and the subfamily *Nepetoideae* of the *Lamiaceae*. Nevertheless, rosmarinic acid can also be found in species of other higher plant families and in some fern and hornwort species (Petersen, 2003). There is ample evidence that rosmarinic acid has beneficial health-promoting effects, such as antiviral, antibacterial, anti-inflammatory and antioxidant properties (Kim et al., 2015). In plants, rosmarinic acid is thought to perform as a preformed constitutively accumulated defence compound (Petersen, 2003). Rosmarinic acid was first isolated and purified in 1958 from *Rosmarinus officinalis* by two Italian chemists, Scarpati and Oriente. The biosynthesis of rosmarinic acid has been extensively studied, and a biosynthetic pathway was first reported in 1970 by Ellis and Towers. They demonstrated that two aromatic amino acids, L-tyrosine

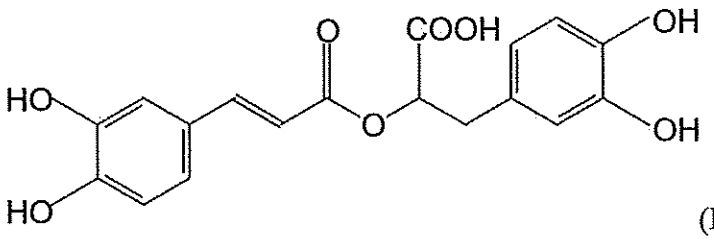
and L-phenylalanine, are the building blocks of rosmarinic acid (Figure 2.3) (Al-Dhabi et al., 2014). Rosmarinic acid, having the molar mass of 360 g/mol, is rapidly eliminated from the blood circulation after intravenous administration and shows very low toxicity, with a LD50 in mice of 561 mg/kg after intravenous application (Petersen, 2003). Certain studies have been performed on rosmarinic acid and its biological and pharmacological activities.

Due to the above described problems in the art, the present invention seeks to provide novel therapeutic approaches for the treatment of metabolic diseases such as diabetes types I and II, the metabolic syndrome and obesity.

The above problem is solved in a first aspect by a method for inhibiting a lipase, the method comprising contacting said lipase with an effective amount of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof.

In course of the present invention it was surprisingly found that plant extracts containing rosmarinic acid have an activity towards inhibition of pancreatic lipase. The present invention shows that rosmarinic acid is a strong lipase inhibitor by non-competitive binding to the enzyme outside of its catalytic pocket.

The term "rosmarinic acid" as used herein refers to a carboxylic acid known in the chemical name "(2R)-2-[[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl)propanoic acid". Rosmarinic acid can be described by the chemical structure I:



(I)

These terms and the drawing are synonymous and can be used interchangeably. As used herein, the term "rosmarinic acid" includes salts of rosmarinic acid base. Any suitable salt of rosmarinic acid can be used in accordance with the invention. Structurally, rosmarinic acid is a dimer of caffeic acid.

Rosmarinic acid can be derived from plants such as rosemary (Rosmarinus officinalis), oregano (*Origanum sp.*), thyme (*thymus sp.*), sage (*Salvia officinalis*), peppermint (a sterile cross of *Mentha aquatic*a and *Mentha spicata*) as well as other plants. In humans, rosmarinic acid is understood to be metabolized into, inter alia, methylated rosmarinic acid, coumaric acid, ferulic acid and caffeic acid.

Rosmarinic acid is a naturally occurring (e.g. plant derived) polyphenol. The rosmarinic acid component can be for example a plant or a portion thereof (e.g. leaves) comprising a rosmarinic acid active ingredient, for example comprising at least 2% rosmarinic acid, for example plants or portions of *Rosmarinus officinalis, Mentha spicata, Origanum sp, Thymus sp. Prunella sp. Melissa, Salvia* or *Perilla* plants and/or combinations thereof, including for example dried, crushed and/or ground plant, leaves or the like; or an extract (i.e. the rosmarinic acid component can be comprised in an extract), such as a plant extract, for example an extract of *Rosmarinus officinalis, Mentha spicata, Origanum sp, Thymus sp. Prunella sp. Melissa, Salvia*, or *Perilla* and/or combinations thereof, comprising for example, at least 2% rosmarinic acid active ingredient. Additional plant species are known to comprise rosmarinic acid and are also suitable for use with the compositions and methods of the disclosure. The extract can be an extract of the whole plant, or a part such as the leaves. The rosmarinic acid component can be a powder, solution, or mixture and can be natural.

A lipase in context of the present disclosure is preferably a pancreatic lipase.

An effective amount of rosmarinic acid in context with the present invention is an amount ranging from 5 to 500 µg/ml, preferably of 10 to 100 µg/ml, and preferably is 15 to 25µg/ml.

The method of the invention is in some embodiments an *in vitro* or *ex vivo* method.

The term "inhibition" in this context is an inhibition of the enzymatic catalysis of hydrolysis of a fat molecule by said lipase enzyme. Preferably the inhibition is non-competitive.

When said lipase is contacted with the rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, the secondary structure of the lipase is altered which causes a reduction of the enzymatic activity of the lipase.

The problem of the invention is also solved in a second aspect by a method for treating a metabolic disorder, the method comprising the administration of a therapeutically effective amount of rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, to a patient in need of such a treatment.

A metabolic disorder in context of the present invention shall be preferably a disorder characterized by aberrant whole-body glucose, lipid and/or protein metabolism of a species and pathological consequences arising therefrom. The "key elements" of these metabolic disorders include but are not limited to, Type 2 diabetes, prediabetes (impaired fasting glucose or impaired glucose tolerance), metabolic syndrome or indices (key elements) thereof (increased waist circumference, increased fasting plasma glucose, increased fasting plasma triglycerides, decreased fasting high density lipoprotein level, increased blood pressure), insulin resistance, hyperinsulinemia, cardiovascular disease (or key elements thereof such as arteriosclerosis, coronary artery disease, peripheral vascular disease, or cerebrovascular disease), congestive heart failure, obesity, elevated plasma norepinephrine, elevated cardiovascular-related inflammatory factors, elevated plasma factors potentiating vascular endothelial dysfunction, hyperlipoproteinemia, arteriosclerosis or atherosclerosis, hyperphagia, hyperglycemia, hyperlipidemia, and hypertension or high blood pressure, increased plasma postprandial triglyceride or free fatty acid levels, increased cellular oxidative stress or plasma indicators thereof, increased circulating hypercoagulative state, renal disease including renal failure and renal insufficiency.

Alternatively provided is rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, for use in the treatment of a metabolic disorder, as defined herein above.

Another aspect of the invention provides a pharmaceutical composition comprising rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, together with a pharmaceutically acceptable carrier and/or excipient. These compositions are preferably for use in the above mentioned methods.

Pharmaceutical compositions may be formulated as tablets, pills, solutions and any other known and suitable pharmaceutical formulations.

The compositions for use in the methods of the herein described invention also include non-pharmaceutical compositions such as cosmetic compositions and food compositions.

Food compositions comprising rosmarinic acid or derivatives or salts thereof, maybe in the form of complete nutritional foods, drinks, mineral waters, soups, food supplements and replacement foods, solutions, sprays, powders, tablets, capsules, nutritional bars, liquid bacterial suspensions, confectionery, milk-based or fermented milk-based products, yogurts, milk-based powders, enteral nutrition products, compositions for children and/or infants, cereal-based products or fermented cereal-based products, soy-based products, ice creams, chocolate, coffee, "culinary" products such as mayonnaise, tomato puree or salad dressings, pet food etc. Thus, the composition may also be intended for animals.

For ingestion, many embodiments of oral compositions and in particular of food supplements are possible. They are formulated by means of the usual methods for producing sugar-coated tablets, gelatine capsules, gels, emulsions, tablets, capsules or solutions. In particular, the composition comprising rosmarinic acid and/or a derivative thereof in combination with a hydrolytic enzyme or with a microorganism containing said enzyme may be incorporated into any other forms of food supplements or of enriched foods, for example food bars, or compacted or non-compacted powders. The powders can be diluted with water, in a fizzy drink, dairy products or soy-derived products or can be incorporated into food bars.

Alternatively, the composition may be a topical composition in the form of aqueous, aqueous-alcoholic or oily solutions, of dispersions of the solution type or dispersions of the lotion or serum type, of emulsions that have a liquid or semi-liquid consistency of the milk type, obtained by dispersion of a fatty phase in an aqueous phase (O/W) or vice-versa (W/O), or of suspensions or emulsion that have a soft, semi-solid or solid consistency of the cream, aqueous gel or anhydrous gel type or else of microemulsions, of microcapsules, or microparticles or of vesicular dispersions of ionic and/or non-ionic type.

The compositions of the invention may comprise the usual excipients and constituents, e.g. fatty and/or aqueous constituents, humectifying agents, thickeners, preserving agents, texturing, flavouring and/or coating agents, antioxidants, dyes that are usual in the food and/or topical domain.

In accordance with the described invention rosmarinic acid, or a derivative or salt thereof, is in particular useful in a non-medical method for weight control or reduction in a mammal. Such a use may be a medial or purely cosmetic use.

Also provided is a method for body fat control, the method comprising a step of administration of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, to a subject. A subject is preferably a mammal, most preferably a human.

Body fat control in context of the invention is a reduction of body fat, or a reduction of an increase in body fat.

The method of the invention may comprise the concomitant intake of food and the rosmarinic acid, or the derivative or the pharmaceutically acceptable salt thereof.

Furthermore, the invention relates to a diet, the diet comprising the intake or administration of an effective amount of rosmarinic acid, or derivative or pharmaceutically acceptable salt thereof. The diet may further comprise additional nutritional rule such as a reduced total calorie intake, reduced carbohydrate or fat proportions of the general macro nutrient food composition of a subject.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the invention within the principles and scope of the broadest interpretations and equivalent configurations thereof.

Description of the Drawings

Figure 1: IC_{50} value of pure RA isolated from O. stamineus. The anti-lipase activity was defined as inhibition percentage of pancreatic lipase in lipase-inhibitory assay. Data were presented as the mean standard deviation ($n \geq 3$).

Figure 2: Inhibition mode determination of RA isolated from O. stamineus. Hanes-Woolf plot; [S]/v versus [S] of kinetic analysis for PPL at two different concentrations of RA. PPL without RA served as control. Data are the result from an average of three experiments (n=3).

Figure 3: Molecular docking of PPL - CLP with RA. Best-docked conformations of PPL-CLP–RA complexes; RA was in ball style, circled in grey. The catalytic sites of PPL (Ser153, Asp177 and His 264) (Hermoso et al., 1996) was circled in purple. Two-dimensional illustrations of the amino acid residues interacting with RA were created using LigPlot. Cluster 1 derived from blind docking with the binding energy of 7.20 kcal/mol.

Detailed Description of the Invention

Materials and Methods

In in vitro lipase-inhibitory assay, rosmarinic acid was incubated with porcine pancreatic lipase (PPL) for 10 minutes at 37°C prior addition of substrate. Inhibition of the lipase activity was expressed as the percentage decrease in the activity when PPL was incubated with rosmarinic acid. Lipase inhibition (%) was calculated according to the following formula:

$$\textbf{Lipase inhibition (I\%)} = 100 - [(B - b)/ (A - a) \times 100]$$

where A is the activity without inhibitor, a is the negative control without inhibitor, B is the activity with inhibitor and b is the negative control with inhibitor.

Example 1: IC_{50} value of rosmarinic acid (RA)

The findings from IC_{50} value showed that RA markedly inhibited PPL activity in a dose-dependent manner (Figure 1). RA gave an IC50 value of 19.5 µg/ml, which was about 2.3 fold stronger than O. stamineus crude extract (44.05 µg/ml). This was because RA occurred in pure and concentrated form if compared to the crude extract, wherein both active and non-active compounds were mixed together in the crude extract. Since the IC_{50} value was lower and better when RA was present in pure form, it was believed that antagonistic action occurred in the crude extract which caused a reduction in the lipase inhibition activity. However, pure RA and O. stamineus crude extract were less potent than orlistat (control) in inhibiting pancreatic lipase. Orlistat gave an IC_{50} value of 1.4 µg/ml, which was about 14 times stronger than pure RA.

Example 2: Inhibition mode of rosmarinic acid

The inhibition mode of RA was visualized using graphical representation of the Michaelis–Menten equation, Hanes-Woolf plot; [S]/v versus [S] as shown in Figure 2. The graph depicts that when the concentration of RA is increased, the value of the y-intercept in the equation for each curve

increased, whereas the x-intercept remained at fixed point showing these inhibitors did not affect Km but the Vmax decreased. Km value for PPL was 0.76 mM and Vmax was 0.0058 mM/minute. A kinetic study in the presence of RA showed reduction of Vmax to 0.0041 mM/minute while the Vmax remain unchanged. Therefore, the enzyme kinetics result showed that RA exerted an inhibitory effect on pancreatic lipase in a noncompetitive manner and therefore binds to the enzyme not at the catalytic center but at a different site. Accordingly, RA inhibited pancreatic lipase by binding with the free enzyme or the enzyme–substrate complex.

Example 3: Molecular Docking

Prediction of the RA potential inhibition site towards PPL was performed using *In silico* experimental approach. The molecular docking task was conducted using the Yet Another Scientific Artificial Reality Application (YASARA) Structure package; an easy-to-use, reliable, universal package for molecular graphics, molecular modeling and molecular dynamics (http://www.yasara.com) (Krieger *et al.*, 2002). The RA structure was obtained from the National Center for Biotechnology Information (NCBI) PubChem with the entry no CID 44258657. The PPL model was obtained from the Protein Data Bank (PDB) crystal structure of the PPL-CLP-TGME (PDB-ID: 1ETH). RA was considered fully flexible and PPL-CLP was considered rigid during docking. The PPL-CLP structure soaked in water was optimized with the YASARA energy minimization macro (em_run.mcr) using the AMBER03 force field (Duan *et al.*, 2003). The resulting structure of PPL-CLP was used as a target for the blind-docking of RA to detect possible cavities that might serve as a binding site in this study. All of the docking calculations were set using a YASARA macro (dock_run.mcr). Other docking parameters used in this analysis were as follows: 25 docking runs; 25,000,000 energy evaluations; 150 population sizes; the number of generation were 27,000. The generated solutions were ranked based on score free binding energy. After docking simulation, the binding energy was obtained from the summary of log file. The data was sorted by the positioned distances of oxidizable carbon atom and binding energy, where shorter distance and positive energy indicated stronger binding. On locating the potential binding sites, docked complex conformations were analysed by their fit within the receptor pocket for continuum and discrete Van der Waals interaction, hydrogen bonding, hydrophobicity, electrostatics and entropy.

In YASARA, the cluster conformation was sorted by binding energy. More positive energies indicated stronger binding while negative energies meant no binding. Based on the docking prediction result, there was a possible binding of ligand to the PPL non-active site. Blind docking of RA to PPL-CLP complex resulted in 25 different cluster conformations. They were differed by at least 5.0 Å heavy atoms RMSD. Cluster 1 with the binding energy of 7.20 kcal/mol showed that RA contacted with 22 amino acids which are Asp 206, Ala 207, Ala 208, Pro 209, Asn 213, Leu 214, Phe 216, Lys 233, Gln 234, Cys 238, Gln 239, Lys 240, Asn 241, Ile 242, Gln 245, Asp 258, Phe 259, Val 260, Ala 261, Cys 262, Asn 263, and His 264. Residues that formed hydrogen bonding with RA are Lys 233, Cys 238, Gln 239, Lys 240, and Asn 263. These hydrogen bonding interactions along with other non-binding interactions played an important role in increasing binding affinity of RA to lipase [Figure 3]. Cluster 1 showed the preferred binding site of RA on the PPL wherein it demonstrated the highest binding energy which proportional to the strongest binding towards PPL residues. Inhibition mode study revealed a non-competitive inhibition of lipase by RA. This meant that RA did not bind to the PPL catalytic site. Hence, docking findings was in agreement with the inhibition mode result. It was predicted that the interaction of RA and PPL was non-covalent. Theoretically, non-covalent interactions between phenolic compounds and proteins are hydrophobic in nature and may stabilize with hydrogen binding (Wu *et al.*, 2014). These non-covalent–bond interactions may alter the enzyme molecular conformation. The conformational mobility of the protein structure may influence the catalytic activity of enzymes (Sinkovits *et al.*, 2007).

Claims

1. A method for inhibiting a lipase, the method comprising contacting said lipase with an effective amount of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof.

2. The method according to claim 1, wherein the lipase is a pancreatic lipase.

3. The method according to claim 1 or 2, wherein the effective amount of rosmarinic acid is an amount ranging from 5 to 500 µg/ml, preferably of 10 to 100 µg/ml, and preferably is 15 to 25µg/ml.

4. The method according to any of claims 1 to 3, wherein the method is an *in vitro* method.

5. The method according to any of claims 1 to 4, wherein the inhibition is an inhibition of the enzymatic catalysis of hydrolysis of a fat molecule by said lipase enzyme.

6. The method according to any of claims 1 to 5, wherein the inhibition of said lipase in non-competitive

7. The method according to any of claims 1 to 6, wherein upon contacting said lipase with the rosmarinic acid, the secondary structure of the lipase changes.

8. The method according to any of claims 1 to 7, wherein the rosmarinic acid upon contacting said lipase binds not at the active site for the enzymatic function of said lipase.

9. A method for treating a metabolic disorder, the method comprising the administration of a therapeutically effective amount of rosmarinic acid to a patient in need of such a treatment.

10. The method according to claim 9, wherein the metabolic disorder is obesity, diabetes type I or II, the metabolic syndrome or related disorders.

11. Rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, for use in the treatment of a metabolic disorder.

12. The rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, according to claim 11, for use in the treatment of obesity, diabetes type I or II, the metabolic syndrome or related disorders.

13. A pharmaceutical composition comprising rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, together with a pharmaceutically acceptable carrier and/or excipient.

14. The pharmaceutical composition according to claim 13, for use in the treatment of a metabolic disorder, such as obesity diabetes type I or II, the metabolic syndrome or related disorders.

15. Use of rosmarinic acid, or a derivative or salt thereof, for use in a non-medical method for weight control or reduction in a mammal.

16. The use according to claim 15, wherein the use is a cosmetic use.

17. A method for body fat control, the method comprising a step of administration of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, to a subject.

18. The method according to claim 17, wherein the body fat control is a reduction of body fat, or a reduction of an increase in body fat.

19. The method according to claim 17 or 18, wherein the subject is a mammal, preferably a human.

20. The method according to any of claims 17 to 19, wherein the method comprises the concomitant intake of food and the rosmarinic acid, or the derivative or the pharmaceutically acceptable salt thereof.

21. A diet, the diet comprising the intake or administration of an effective amount of rosmarinic acid, or derivative or pharmaceutically acceptable salt thereof.

22. Use of rosmarinic acid, or derivative or pharmaceutically acceptable salt thereof, in a diet for inhibiting fat metabolism.

Abstract

The present invention pertains to rosmarinic acid for use in the inhibition of pancreactic lipase. The use of the present invention may be applied in medical and dietary methods for the reduction of body fat in mammals. The invention is in particular useful for the treatment of metabolic disorders such as obesity or diabetes.

ROSMARINIC ACID AS PANCREACTIC LIPASE INHIBITOR

Field of the Invention

The present invention pertains to rosmarinic acid for use in the inhibition of pancreatic lipase. The use of the present invention may be applied in medical and dietary methods for the reduction of body fat in mammals. The invention is in particular useful for the treatment of metabolic disorders such as obesity or diabetes.

Background of the Invention

Obesity is defined as an excessive fat accumulation detrimental to human health. It has also been defined as increased adipose tissue mass as a result of an enlargement in fat cells or an increase in their number (Couillard et al., 2000). A crude measure of obesity is the Body Mass Index (BMI), calculated as body weight in kilogram divided by the square of height in meters. Overweight is defined as a BMI of 25.0–29.9 kg/m2, and a BMI exceeding 30 kg/m2 is considered as obese. Extreme obesity is defined as a BMI of greater than 40 kg/m2 (Thompson et al., 2007). However, there is a demand for a more limited range for BMI classification in Asia because of the high prevalence of comorbidities, particularly diabetes and hypertension (James et al., 2001). Another approach to assessing obesity is measuring waist to hip ratio (WHR), which is a better indicator of other severe health conditions. The National Institute of Diabetes, Digestive, and Kidney Diseases, USA, has indicated that men with waist–hip ratios of more than 1.0 and women with more than 0.8 are at greater health risk as a consequence of their fat distribution.

Lipases are enzymes that digest fats, including triacylglycerol and phospholipids. Human lipases include the pre-duodenal (lingual and gastric) and the extra-duodenal (pancreatic, hepatic, LPL and the endothelial) lipases (Mukherjee, 2003). Pancreatic lipase (triacylglycerol acyl hydrolase), the principal lipolytic enzyme, plays a key role in the efficient digestion of triglycerides. It removes fatty acids from the α and α' position of dietary triglycerides, yielding β-monoglycerides and long chain saturated and polyunsaturated fatty acids as lipolytic products (Mukherjee, 2003; Shi and Burn, 2004; Thomson et al., 1997). Pancreatic lipase, encoded by the

PNLIP gene in humans is secreted into the duodenum through the duct system of the pancreas. Pancreatic lipase is responsible for the hydrolysis of 50–70% of total dietary fats (Birari and Bhutani, 2007). The primary structure of the pancreatic lipase was determined by analysis of recombinant cDNA clones derived from a cDNA library of human pancreas. The sequence was found to be a single chain glycoprotein of 449 amino acids while the three-dimensional structure of human pancreatic lipase was solved by X-ray crystallography. The encoded protein shows 86% and 68% homology with porcine and canine pancreatic lipase, respectively (Thomson et al., 1997).

Pancreatic lipase inhibition is one of the well-known modes of action for the determination of the potential efficacy of natural products as antiobesity agents (Sugiyama et al., 2007). Currently, the only lipase inhibitor available in the market which has obtained approval by the FDA for obesity treatment is tetrahydrolipstatin, a chemically-derived analogue of lipstatin (Weibel et al., 1987) from Hoffmann La Roche (Basel, Switzerland). It is commonly known as orlistat and is marketed as Xenical® (Manufacturer: Roche) or alli® (Manufacturer: GlaxoSmithKline). In Malaysia, orlistat is accessible by a brand name Cuvarlix®, which is marketed by Pharmaniaga (Figure 2.1). Orlistat was derived from lipstatin which was isolated from the mycelium of Streptomyces toxytricini (a soil microbe) in 1987 by Weibel et al. Orlistat inhibits pancreatic lipase in an irreversible manner with an IC50 of 0.14 μM. Orlistat contains a β-lactone moiety that is essential for activity (Weibel et al., 1987). Orlistat is the example of successful pancreatic lipase inhibitor, up to now. However, it has several gastrointestinal side effects such as oily stools, oily spotting, and flatulence (Birari and Bhutani, 2007). Due to these reasons, many researchers have attempted to obtain newer pancreatic lipase inhibitors that lack these unpleasant side effects.

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is commonly found in species of *Boraginaceae* and the subfamily *Nepetoideae* of the *Lamiaceae*. Nevertheless, rosmarinic acid can also be found in species of other higher plant families and in some fern and hornwort species (Petersen, 2003). There is ample evidence that rosmarinic acid has beneficial health-promoting effects, such as antiviral, antibacterial, anti-inflammatory and antioxidant properties (Kim et al., 2015). In plants, rosmarinic acid is thought to perform as a preformed constitutively accumulated defence compound (Petersen, 2003). Rosmarinic acid was first isolated and purified in 1958 from *Rosmarinus officinalis* by two Italian chemists, Scarpati

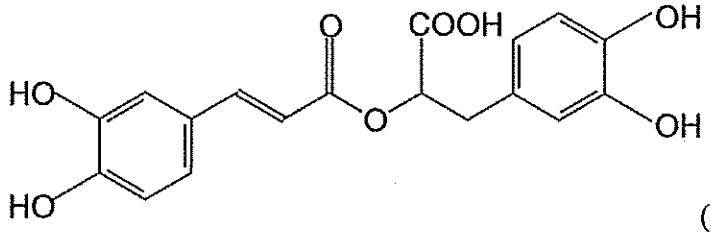
and Oriente. The biosynthesis of rosmarinic acid has been extensively studied, and a biosynthetic pathway was first reported in 1970 by Ellis and Towers. They demonstrated that two aromatic amino acids, L-tyrosine and L-phenylalanine, are the building blocks of rosmarinic acid (Figure 2.3) (Al-Dhabi et al., 2014). Rosmarinic acid, having the molar mass of 360 g/mol, is rapidly eliminated from the blood circulation after intravenous administration and shows very low toxicity, with a LD50 in mice of 561 mg/kg after intravenous application (Petersen, 2003). Certain studies have been performed on rosmarinic acid and its biological and pharmacological activities.

Due to the above described problems in the art, the present invention seeks to provide novel therapeutic approaches for the treatment of metabolic diseases such as diabetes types I and II, the metabolic syndrome and obesity.

The above problem is solved in a first aspect by a method for inhibiting a lipase, the method comprising contacting said lipase with an effective amount of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof.

In course of the present invention it was surprisingly found that plant extracts containing rosmarinic acid have an activity towards inhibition of pancreatic lipase. The present invention shows that rosmarinic acid is a strong lipase inhibitor by non-competitive binding to the enzyme outside of its catalytic pocket.

The term "rosmarinic acid" as used herein refers to a carboxylic acid known in the chemical name "(2R)-2-[[(2E)-3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxyphenyl)propanoic acid". Rosmarinic acid can be described by the chemical structure I:



(I)

These terms and the drawing are synonymous and can be used interchangeably. As used herein, the term "rosmarinic acid" includes salts of rosmarinic acid base. Any suitable salt of rosmarinic acid can be used in accordance with the invention. Structurally, rosmarinic acid is a dimer of caffeic acid. Rosmarinic acid can be derived from plants such as rosemary (Rosmarinus officinalis), oregano (*Origanum sp.*), thyme (*thymus sp.*), sage (*Salvia officinalis*), peppermint (a sterile cross of *Mentha aquatic*a and *Mentha spicata*) as well as other plants. In humans, rosmarinic acid is understood to be metabolized into, inter alia, methylated rosmarinic acid, coumaric acid, ferulic acid and caffeic acid.

Rosmarinic acid is a naturally occurring (e.g. plant derived) polyphenol. The rosmarinic acid component can be for example a plant or a portion thereof (e.g. leaves) comprising a rosmarinic acid active ingredient, for example comprising at least 2% rosmarinic acid, for example plants or portions of *Rosmarinus officinalis*, *Mentha spicata*, *Origanum sp*, *Thymus sp. Prunella sp. Melissa*, *Salvia* or *Perilla* plants and/or combinations thereof, including for example dried, crushed and/or ground plant, leaves or the like; or an extract (i.e. the rosmarinic acid component can be comprised in an extract), such as a plant extract, for example an extract of *Rosmarinus officinalis*, *Mentha spicata*, *Origanum sp*, *Thymus sp. Prunella sp. Melissa*, *Salvia*, or *Perilla* and/or combinations thereof, comprising for example, at least 2% rosmarinic acid active ingredient. Additional plant species are known to comprise rosmarinic acid and are also suitable for use with the compositions and methods of the disclosure. The extract can be an extract of the whole plant, or a part such as the leaves. The rosmarinic acid component can be a powder, solution, or mixture and can be natural.

A lipase in context of the present disclosure is preferably a pancreatic lipase.

An effective amount of rosmarinic acid in context with the present invention is an amount ranging from 5 to 500 µg/ml, preferably of 10 to 100 µg/ml, and preferably is 15 to 25µg/ml.

The method of the invention is in some embodiments an *in vitro* or *ex vivo* method.

The term "inhibition" in this context is an inhibition of the enzymatic catalysis of hydrolysis of a fat molecule by said lipase enzyme. Preferably the inhibition is non-competitive.

When said lipase is contacted with the rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, the secondary structure of the lipase is altered which causes a reduction of the enzymatic activity of the lipase.

The problem of the invention is also solved in a second aspect by a method for treating a metabolic disorder, the method comprising the administration of a therapeutically effective amount of rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, to a patient in need of such a treatment.

A metabolic disorder in context of the present invention shall be preferably a disorder characterized by aberrant whole-body glucose, lipid and/or protein metabolism of a species and pathological consequences arising therefrom. The "key elements" of these metabolic disorders include but are not limited to, Type 2 diabetes, prediabetes (impaired fasting glucose or impaired glucose tolerance), metabolic syndrome or indices (key elements) thereof (increased waist circumference, increased fasting plasma glucose, increased fasting plasma triglycerides, decreased fasting high density lipoprotein level, increased blood pressure), insulin resistance, hyperinsulinemia, cardiovascular disease (or key elements thereof such as arteriosclerosis, coronary artery disease, peripheral vascular disease, or cerebrovascular disease), congestive heart failure, obesity, elevated plasma norepinephrine, elevated cardiovascular-related inflammatory factors, elevated plasma factors potentiating vascular endothelial dysfunction, hyperlipoproteinemia, arteriosclerosis or atherosclerosis, hyperphagia, hyperglycemia, hyperlipidemia, and hypertension or high blood pressure, increased plasma postprandial triglyceride or free fatty acid levels, increased cellular oxidative stress or plasma indicators thereof, increased circulating hypercoagulative state, renal disease including renal failure and renal insufficiency.

Alternatively provided is rosmarinic acid, or derivatives or a pharmaceutically acceptable salt thereof, for use in the treatment of a metabolic disorder, as defined herein above.

Another aspect of the invention provides a pharmaceutical composition comprising rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, together with a pharmaceutically

acceptable carrier and/or excipient. These compositions are preferably for use in the above mentioned methods.

Pharmaceutical compositions may be formulated as tablets, pills, solutions and any other known and suitable pharmaceutical formulations.

The compositions for use in the methods of the herein described invention also include non-pharmaceutical compositions such as cosmetic compositions and food compositions.

Food compositions comprising rosmarinic acid or derivatives or salts thereof, maybe in the form of complete nutritional foods, drinks, mineral waters, soups, food supplements and replacement foods, solutions, sprays, powders, tablets, capsules, nutritional bars, liquid bacterial suspensions, confectionery, milk-based or fermented milk-based products, yogurts, milk-based powders, enteral nutrition products, compositions for children and/or infants, cereal-based products or fermented cereal-based products, soy-based products, ice creams, chocolate, coffee, "culinary" products such as mayonnaise, tomato puree or salad dressings, pet food etc. Thus, the composition may also be intended for animals.

For ingestion, many embodiments of oral compositions and in particular of food supplements are possible. They are formulated by means of the usual methods for producing sugar-coated tablets, gelatine capsules, gels, emulsions, tablets, capsules or solutions. In particular, the composition comprising rosmarinic acid and/or a derivative thereof in combination with a hydrolytic enzyme or with a microorganism containing said enzyme may be incorporated into any other forms of food supplements or of enriched foods, for example food bars, or compacted or non-compacted powders. The powders can be diluted with water, in a fizzy drink, dairy products or soy-derived products or can be incorporated into food bars.

Alternatively, the composition may be a topical composition in the form of aqueous, aqueous-alcoholic or oily solutions, of dispersions of the solution type or dispersions of the lotion or serum type, of emulsions that have a liquid or semi-liquid consistency of the milk type, obtained by dispersion of a fatty phase in an aqueous phase (O/W) or vice-versa (W/O), or of suspensions or emulsion that have a soft, semi-solid or solid consistency of the cream, aqueous gel or

anhydrous gel type or else of microemulsions, of microcapsules, or microparticles or of vesicular dispersions of ionic and/or non-ionic type.

The compositions of the invention may comprise the usual excipients and constituents, e.g. fatty and/or aqueous constituents, humectifying agents, thickeners, preserving agents, texturing, flavouring and/or coating agents, antioxidants, dyes that are usual in the food and/or topical domain.

In accordance with the described invention rosmarinic acid, or a derivative or salt thereof, is in particular useful in a non-medical method for weight control or reduction in a mammal. Such a use may be a medial or purely cosmetic use.

Also provided is a method for body fat control, the method comprising a step of administration of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof, to a subject. A subject is preferably a mammal, most preferably a human.

Body fat control in context of the invention is a reduction of body fat, or a reduction of an increase in body fat.

The method of the invention may comprise the concomitant intake of food and the rosmarinic acid, or the derivative or the pharmaceutically acceptable salt thereof.

Furthermore, the invention relates to a diet, the diet comprising the intake or administration of an effective amount of rosmarinic acid, or derivative or pharmaceutically acceptable salt thereof. The diet may further comprise additional nutritional rule such as a reduced total calorie intake, reduced carbohydrate or fat proportions of the general macro nutrient food composition of a subject.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the invention within the principles and scope of the broadest interpretations and equivalent configurations thereof.

Description of the Drawings

Figure 1: IC_{50} value of pure RA isolated from O. stamineus. The anti-lipase activity was defined as inhibition percentage of pancreatic lipase in lipase-inhibitory assay. Data were presented as the mean standard deviation ($n \geq 3$).

Figure 2: Inhibition mode determination of RA isolated from O. stamineus. Hanes-Woolf plot; [S]/v versus [S] of kinetic analysis for PPL at two different concentrations of RA. PPL without RA served as control. Data are the result from an average of three experiments (n=3).

Figure 3: Molecular docking of PPL - CLP with RA. Best-docked conformations of PPL-CLP–RA complexes; RA was in ball style, circled in grey. The catalytic sites of PPL (Ser153, Asp177 and His 264) (Hermoso et al., 1996) was circled in purple. Two-dimensional illustrations of the amino acid residues interacting with RA were created using LigPlot. Cluster 1 derived from blind docking with the binding energy of 7.20 kcal/mol.

Detailed Description of the Invention

Materials and Methods

In in vitro lipase-inhibitory assay, rosmarinic acid was incubated with porcine pancreatic lipase (PPL) for 10 minutes at 37°C prior addition of substrate. Inhibition of the lipase activity was expressed as the percentage decrease in the activity when PPL was incubated with rosmarinic acid. Lipase inhibition (%) was calculated according to the following formula:

$$\textbf{Lipase inhibition (I\%)} = 100 - [(B - b)/(A - a) \times 100]$$

where A is the activity without inhibitor, a is the negative control without inhibitor, B is the activity with inhibitor and b is the negative control with inhibitor.

Example 1: IC_{50} value of rosmarinic acid (RA)

The findings from IC_{50} value showed that RA markedly inhibited PPL activity in a dose-dependent manner (Figure 1). RA gave an IC50 value of 19.5 µg/ml, which was about 2.3 fold stronger than O. stamineus crude extract (44.05 µg/ml). This was because RA occurred in pure and concentrated form if compared to the crude extract, wherein both active and non-active compounds were mixed together in the crude extract. Since the IC_{50} value was lower and better when RA was present in pure form, it was believed that antagonistic action occurred in the crude extract which caused a reduction in the lipase inhibition activity. However, pure RA and O. stamineus crude extract were less potent than orlistat (control) in inhibiting pancreatic lipase. Orlistat gave an IC_{50} value of 1.4 µg/ml, which was about 14 times stronger than pure RA.

Example 2: Inhibition mode of rosmarinic acid

The inhibition mode of RA was visualized using graphical representation of the Michaelis–Menten equation, Hanes-Woolf plot; [S]/v versus [S] as shown in Figure 2. The graph depicts that when the concentration of RA is increased, the value of the y-intercept in the equation for

each curve increased, whereas the x-intercept remained at fixed point showing these inhibitors did not affect Km but the Vmax decreased. Km value for PPL was 0.76 mM and Vmax was 0.0058 mM/minute. A kinetic study in the presence of RA showed reduction of Vmax to 0.0041 mM/minute while the Vmax remain unchanged. Therefore, the enzyme kinetics result showed that RA exerted an inhibitory effect on pancreatic lipase in a noncompetitive manner and therefore binds to the enzyme not at the catalytic center but at a different site. Accordingly, RA inhibited pancreatic lipase by binding with the free enzyme or the enzyme–substrate complex.

Example 3: Molecular Docking

Prediction of the RA potential inhibition site towards PPL was performed using *In silico* experimental approach. The molecular docking task was conducted using the Yet Another Scientific Artificial Reality Application (YASARA) Structure package; an easy-to-use, reliable, universal package for molecular graphics, molecular modeling and molecular dynamics (http://www.yasara.com) (Krieger *et al.*, 2002). The RA structure was obtained from the National Center for Biotechnology Information (NCBI) PubChem with the entry no CID 44258657. The PPL model was obtained from the Protein Data Bank (PDB) crystal structure of the PPL-CLP-TGME (PDB-ID: 1ETH). RA was considered fully flexible and PPL-CLP was considered rigid during docking. The PPL-CLP structure soaked in water was optimized with the YASARA energy minimization macro (em_run.mcr) using the AMBER03 force field (Duan *et al.*, 2003). The resulting structure of PPL-CLP was used as a target for the blind-docking of RA to detect possible cavities that might serve as a binding site in this study. All of the docking calculations were set using a YASARA macro (dock_run.mcr). Other docking parameters used in this analysis were as follows: 25 docking runs; 25,000,000 energy evaluations; 150 population sizes; the number of generation were 27,000. The generated solutions were ranked based on score free binding energy. After docking simulation, the binding energy was obtained from the summary of log file. The data was sorted by the positioned distances of oxidizable carbon atom and binding energy, where shorter distance and positive energy indicated stronger binding. On locating the potential binding sites, docked complex conformations were analysed by their fit within the receptor pocket for continuum and discrete Van der Waals interaction, hydrogen bonding, hydrophobicity, electrostatics and entropy.

In YASARA, the cluster conformation was sorted by binding energy. More positive energies indicated stronger binding while negative energies meant no binding. Based on the docking prediction result, there was a possible binding of ligand to the PPL non-active site. Blind docking of RA to PPL-CLP complex resulted in 25 different cluster conformations. They were differed by at least 5.0 Å heavy atoms RMSD. Cluster 1 with the binding energy of 7.20 kcal/mol showed that RA contacted with 22 amino acids which are Asp 206, Ala 207, Ala 208, Pro 209, Asn 213, Leu 214, Phe 216, Lys 233, Gln 234, Cys 238, Gln 239, Lys 240, Asn 241, Ile 242, Gln 245, Asp 258, Phe 259, Val 260, Ala 261, Cys 262, Asn 263, and His 264. Residues that formed hydrogen bonding with RA are Lys 233, Cys 238, Gln 239, Lys 240, and Asn 263. These hydrogen bonding interactions along with other non-binding interactions played an important role in increasing binding affinity of RA to lipase [Figure 3]. Cluster 1 showed the preferred binding site of RA on the PPL wherein it demonstrated the highest binding energy which proportional to the strongest binding towards PPL residues. Inhibition mode study revealed a non-competitive inhibition of lipase by RA. This meant that RA did not bind to the PPL catalytic site. Hence, docking findings was in agreement with the inhibition mode result. It was predicted that the interaction of RA and PPL was non-covalent. Theoretically, non-covalent interactions between phenolic compounds and proteins are hydrophobic in nature and may stabilize with hydrogen binding (Wu *et al.*, 2014). These non-covalent–bond interactions may alter the enzyme molecular conformation. The conformational mobility of the protein structure may influence the catalytic activity of enzymes (Sinkovits *et al.*, 2007).

Claims

1. A method for inhibiting a lipase, the method comprising contacting said lipase with an effective amount of rosmarinic acid, or a derivative or pharmaceutically acceptable salt thereof.

2. The method according to claim 1, wherein the lipase is a pancreatic lipase.

3. The method according to claim 1 or 2, wherein the effective amount of rosmarinic acid is an amount ranging from 5 to 500 µg/ml, preferably of 10 to 100 µg/ml, and preferably is 15 to 25µg/ml.

4. The method according to any of claims 1 to 3, wherein the method is an *in vitro* method.

5. The method according to any of claims 1 to 4, wherein the inhibition is an inhibition of the enzymatic catalysis of hydrolysis of a fat molecule by said lipase enzyme.

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7. The method according to any of claims 1 to 6, wherein upon contacting said lipase with the rosmarinic acid, the secondary structure of the lipase changes.

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19. The method according to claim 17 or 18, wherein the subject is a mammal, preferably a human.

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22. Use of rosmarinic acid, or derivative or pharmaceutically acceptable salt thereof, in a diet for inhibiting fat metabolism.

Abstract

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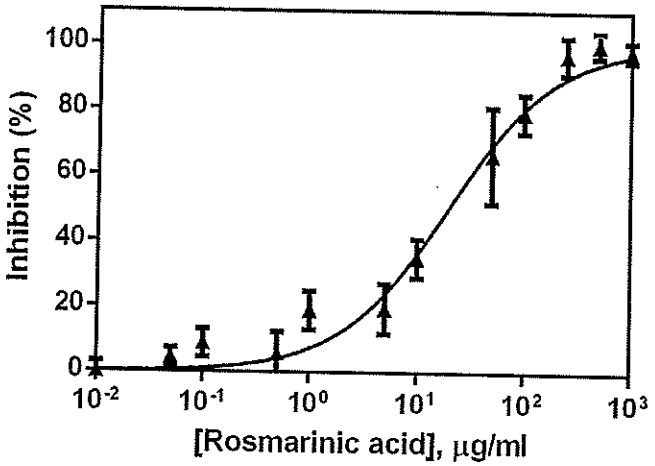
The present invention pertains to rosmarinic acid for use in the inhibition of pancreactic lipase. The use of the present invention may be applied in medical and dietary methods for the reduction of body fat in mammals. The invention is in particular useful for the treatment of metabolic disorders such as obesity or diabetes.

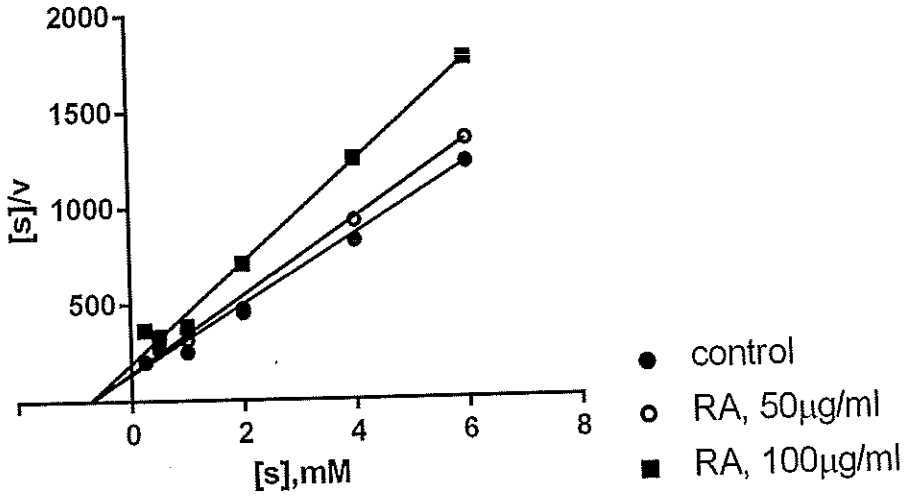
Figures

Figure 1:



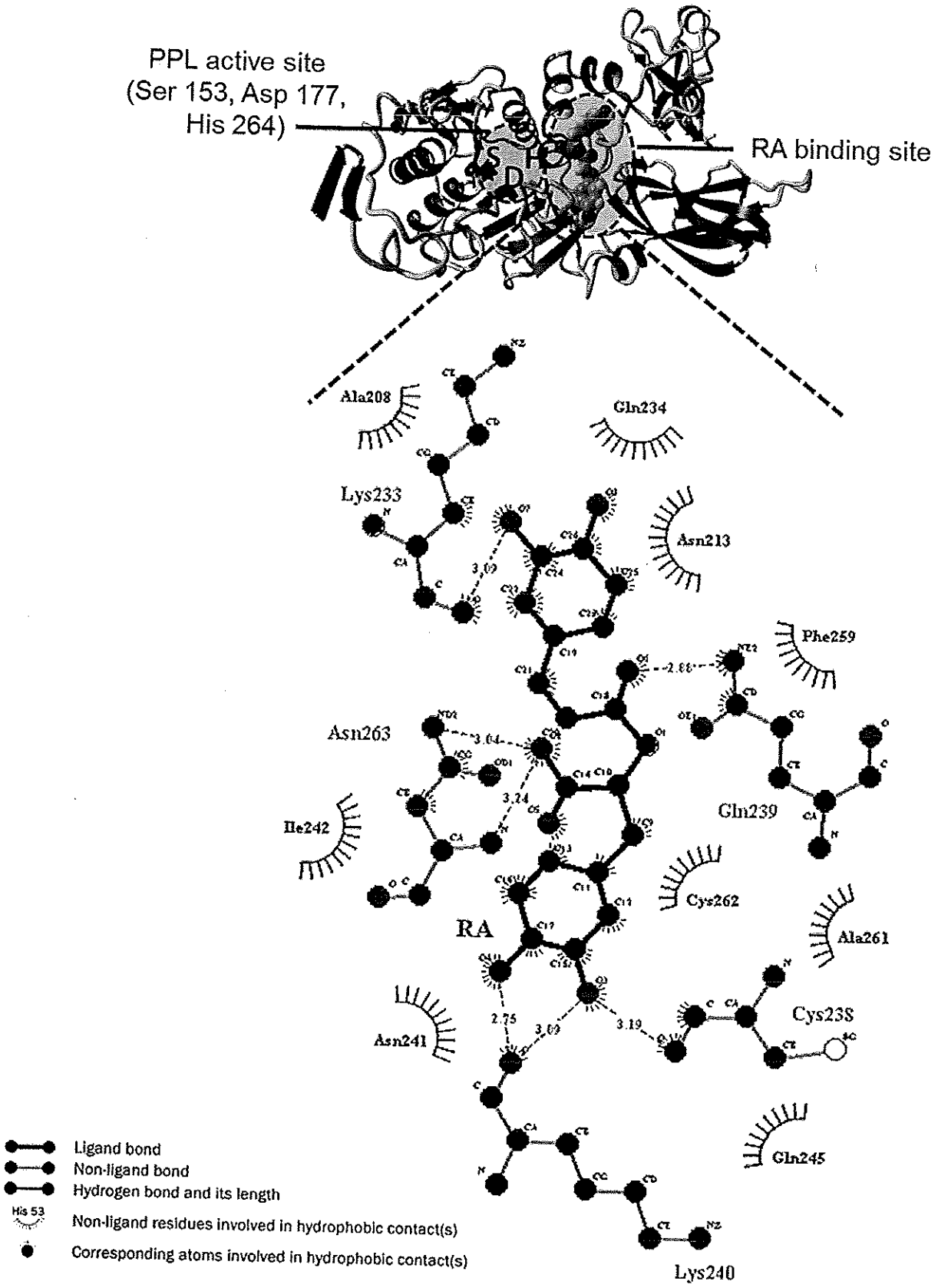
IC_{50}
19.50

Figure 2:



	control	RA, 50µg/ml	RA, 100µg/ml
Vmax	0.005796	0.004092	0.003053
Km	0.7644	0.7421	0.7599

Figure 3:


PPL active site
(Ser 153, Asp 177,
His 264)
RA binding site
Ala208
Gln234
Lys233
Asn213
Phe259
Asn263
Ile242
Gln239
Cys262
Ala261
RA
Asn241
Cys238
Gln245
Lys240
Ligand bond
Non-ligand bond
Hydrogen bond and its length
His 53
Non-ligand residues involved in hydrophobic contact(s)
Corresponding atoms involved in hydrophobic contact(s)