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LIPASE INHIBITORS FROM *NIGELLA SATIVA* AND *PUNICA GRANATUM* AS AN EFFECTIVE APPROACH TOWARDS CONTROLLING OBESITY

Trichur Khabeer Shamsiya
CSIR – Central Food Technological Research Institute (CFTRI), Mysore– 570 020, Karnataka, India, Food Protestants & Infestation Control Department
shamsiya.khabeer@gmail.com,

Manjunatha, J. R
CSIR – Central Food Technological Research Institute (CFTRI), Mysore – 570 020, Karnataka, India Department of Spice & Flavour Science
manjunathajr@cftri.res.in

Manonmani H.K
CSIR – Central Food Technological Research Institute (CFTRI), Mysore – 570 020, Karnataka, India, Food Protestants & Infestation Control Department
manonmani@cftri.res.in

Abstract

Two distinct lipase inhibitor cocktails RAYstat4ns and SHAMstat3pg have been purified from seeds of Nigella sativa and Punica granatum respectively. Structural characterization has shown RAYstat4ns to be a mixture of 3 polyunsaturated fatty acids and one saturated fatty acid while SHAMstat3pg is a triglyceride mixture comprising of three different kinds of polyunsaturated fatty acids. Ethanol extracted fractions of seeds of Nigella saliva and Punic a granatum having high lipase inhibitory activity were purified by HPLC followed by TLC to yield respectively RAYstat4ns and SHAMstat3pg . These plant based lipase inhibitors have shown good lipase inhibitory activity against pancreatic lipase with mixed type of inhibition.

RAYstat4ns and SHAMstat3pg have also shown inhibition of hormone sensitive lipase. The experimental IC_{50} of the purified lipase inhibitors RAYstat4ns and SHAMstat3pg was found to be respectively 4.02µg/µl and 7.35 µg/µl for pancreatic lipase and 6.31 µg/µl and 11.45 µg/µl respectively for hormone sensitive lipase. Hence ,these plant-based lipase inhibitors can be used in controlling lipolysis and insulin resistance in addition to inhibiting absorption of dietary lipid into the living system. In vivo studies could help confirm the potency of these plant based isolates as efficient lipase inhibitors.

Keywords

Lipase Inhibitor, *Nigella Saliva*, *Punica Granatum*, Pancreatic Lipase Inhibitor, Hormone Sensitive Lipase Inhibitor, Anti-Obesity

1. Introduction

Obesity is a globally increasing health problem affecting many classes of people. BMI; comparison of weight to height wherein, obese individuals have a BMI of more than 30 (Organization, W. H., 2003). A high BMI is a reason for many health problems like high blood pressure, dyslipidaemia, cardiovascular diseases, stroke, Type 2 diabetes, high cholesterol joint problems caused by extra weight, sleep apnea, gall stones etc. (Haslam & James, 2005). Obesity is caused by a combination of excessive energy food intake, lack of physical activity, and genetic susceptibility, endocrine disorders, medications, or psychiatric illness (Monasta et al., 2010).

The main treatment for obesity is dieting, physical exercise, bariatric surgery (Lau et al., 2007). Over the counter drugs and medications such as, orlistat (Xenical), lorcaserin (Belviq) and a combination of phentermine and topiramate (Qsymia) are currently being practiced and have evidence for long term applications (Yanovski & Yanovski, 2014). Orlistat, a modified lipase inhibitor of microbial origin (Sambasivarao, 2013) brings about weight loss at an average of 2.9 kg (6.4 lb) at 1 to 4 years. Its use is associated with high rates of gastrointestinal side effects and concerns have been raised about its negative effects on the kidneys.

Pancreatic lipase, the main lipid digesting enzyme, removes fatty acids from the α- and α′- positions of dietary triglycerides, yielding the lipolytic product β-monoglyceride, long chain saturated and polyunsaturated fatty acids. The primary role of lipase inhibitors is to decrease the gastrointestinal absorption of fats and fats are excreted in feces rather than being absorbed to be used as a source of caloric energy. Hence, inhibition of pancreatic lipase is an

attractive targeted approach for the discovery of potent anti-obesity drugs (Thomson, De Pover, Keelan, Jarocka-Cyrta & Clandinin, 1997).

The activity of hormone stimulated lipase (HSL) is acutely activated *via* cAMP and protein kinase A-mediated phosphorylation (Tsujita, Ninomiya, & Okuda, 1989), (Anthonsen, Ronnstrand, Wernstedt & Degerman, 1998) in response to epinephrine/norepinephrine. Insulin stimulation of adipocytes prevents HSL activation, leading to a decrease in the release of free fatty acid (FFA) and glycerol (Claus et al., 2005) .The role of elevated plasma FFA in type 2 diabetes has led to the hypothesis that HSL may be a potential therapeutic target for this disease, by lowering plasma FFA levels and thereby reducing insulin resistance.

In many traditional cultural practices, plants have been used as conventional natural medicines for curing many diseases (Roh & Jung, 2012). This made us to choose plant as an ideal choice to obtain a functional lipase inhibitor, as it could have minimum or nil side effects and its acceptability is noncontroversial. In the search for biologically active pancreatic lipase inhibitor as anti-obesity agents from natural resources, various plant extracts and their phytochemicals like carotenoids, glycosides, polyphenols etc., have been screened for their lipase inhibitory activity (Barma & Goswami, 2013). Apart from these, biologically important Short-chain fatty acids have been shown to inhibit lipolysis (Barma & Goswami, 2013). Nigella sativa oil was found to be effective as add on therapy in patients of insulin resistance syndrome. Nigella sativa oil has a significant activity in diabetic and dyslipidemic patients (Najmi, Nasiruddin, Khan, & Haque, 2008) .The supplementation of pomegranate juice or extracts to diet may prevent or even correct obesity, diabetes, and cardiovascular diseases (Louise, Melo, & Mancini-filho, 2014). Studies have reported that both the pomegranate flowers and fruit extracts exhibited high activity on lowering circulation lipid and modifying heart disease risk factors in diabetic animals and humans with hyperlipidemia. Moreover, Pomegranate juice supplementation to mice with advanced atherosclerosis reduced their macrophage oxidative stress, their macrophage cholesterol flux and even attenuated the development of atherosclerosis (Kaplan et al., 2001). The effects of propionic acid and butyric acid on basal and stimulated lipolysis were studied in primary rat adipocytes (Barma & Goswami, 2013).

Most of the studied lipase inhibitors have lipase inhibitory activity against a single type of lipase. In this study, we have attempted to address a plant based inhibitor having lipase inhibitory activity against pancreatic lipase and hormone sensitive lipase.

2. Materials and Methods

2.1 Plant Collection

Two plants used in this study, ripe fruits of *Punica granatum* of the Bhagwa (Kesar) variety, and *Nigella sativa* seeds were purchased from the local market in Mysore city, Karnataka, India.

2.2 Isolation of Lipase Inhibitor

The fresh ripe pulpy fruit of *Punica granatum* were pressed and strained to remove the juice and pulp. The yellow seeds were separated, washed thoroughly, macerated well and extracted with different solvents such as aqueous, ethanol, methanol and ethyl acetate. The seeds of *Nigella sativa* were ground to a fine powder using a food processor and extracted in a similar way. The extract was filtered with what man no. 1 filter paper .The extraction process was repeated three times and the solvent fractions were pooled. Then the solvent was evaporated to dryness in vacuum evaporator at 37 °C. The different solvent extracts were then resuspended in DMSO and assessed for lipase inhibitory activity.

2.3 Purification and Characterization of the Isolated Lipase Inhibitor

The isolated inhibitor was analyzed using isocratic analytical HPLC, with RP-C18 column [5µm, 250 mm x 4.6 mm, Shimpack CLC-ODS (L1) , LC-10A (Shimadzu, Japan) at a flow rate of 1 ml/ minute and scanned over a UV-Vis spectrum of 210 -320nm. Water: methanol:ethyl acetate (80:15:5) was used as mobile phase.

The peaks as observed in the analytical column were further purified by preparative HPLC applying the same analytical conditions. The individual peaks were collected, evaporated to dryness, resuspended in DMSO and lipase inhibitory assay was carried out. The active peak was further purified by preparative TLC using neutral lipid system, hexane:diethyl ether:acetic acid (6.8:3:0.2) as mobile phase on Silicagel 60 TLC plates (MERCK) (Barma & Goswami, 2013). The plates containing separated spots were air dried and colour was developed in iodine chamber. The fatty acid methyl esters gave brown colored spots, which were marked to calculate R_f values. These separated bands were recovered by preparative TLC, resuspended in DMSO and assessed for lipase inhibitory activity. The fraction with highest lipase inhibitory activity was used for further characterization.

2.4 Identification of Lipase Inhibitor

The purified lipase inhibitors from *Punica granatum* and *Nigella sativa* having highest lipase inhibitory activity were subjected to LC-MS/MS analysis for molecular weight determination in ESI +ve mode. The structure of the purified inhibitor was elucidated using NMR (^{1}H, ^{13}C, COSY, HMBC), FT-IR, GC and GC-MS/MS.

2.4.1 Spectral Characterization

2.4.1.1 NMR Spectra

^{1}H & ^{13}C NMR spectra for the isolated lipase inhibitors samples recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker biospin, Reinstetten, Germany), using $CDCl_3$ solvent. Proton and carbon chemical shifts are given relative to either residual solvent peaks or standard tetramethylsilane. Assignments of ^{1}H and ^{13}C signals were based on ^{1}H, ^{13}C, HMBC (Heteronuclear multiple-bond correlation spectroscopy), DEPT (distortionless enhancement by polarization transfer) and COSY (correlated Spectroscopy) experiments.

2.4.1.2 GC-MS Analysis

Gas chromatography-mass spectrometry [(GC-MS) PerkinElmer (Turbomass Gold) and an GC-flame ionization detector (GC-FID) fitted with TR- FAME column (30.0m x 320µm)] was used for determination of fatty acid composition of purified lipase inhibitor. Helium was used as carrier gas. Fatty acid composition was determined after derivatization of purified lipase inhibitor. 0.2ml KOH 2N in methanol was added to 1ml sample dissolved in 3ml n-hexane. This was vortexed for 2 min till the two phases separate (Topkafa, & Sherazi, 2015). 0.5 microliter of n-hexane (upper phase) was injected into GC-FID. The standard FAME analysis programme was used. The column temperature was set to increase from 130°C (2min) to 280°C (3.57min) @ 7°C/min. The injection was performed with a split ratio 50:1 and constant flow operating mode at 0.8 mL/min (Helium used as carrier gas). The injector temperature is set at 220°C. The injected volume was 1µL. A solvent delay of 4 min was set. The transfer temperature and source temperature was maintained at 200°C and 180°C respectively. The mass spectrometer was operated in the electron impact (EI) mode and scanned at a range of 40 to 400Da. The fatty acid methyl esters were matched with their MS and retention index data with those of the standard's unique spectra and by matching the fragmentation pattern in Mass Spectra with those of NIST 12.LIB.

2.5 Quantification of Lipase Activity by Spectrophotometric Assay

2.5.1 *In Vitro* Pancreatic Lipase Inhibition Enzyme Assay

Pancreatic lipase (PL) inhibitory activity was studied using the substrate *p*-nitrophenyl palmitate (*p*NPP). Purified lipase inhibitors of *Punica granatum* and *Nigella sativa* were assessed separately for their potency as functional lipase inhibitors following a standard colorimetric assay (Adnyana, Sukandar, Yuniarto, & Finna, 2014). The substrate, *p*-nitrophenylpalmitate (*p*NPP) was dissolved in acetonitrile to give a stock solution with a concentration of 50 mM/L. The purified plant lipase inhibitor was dissolved in DMSO (1mg/ml). Porcine pancreatic lipase (type II, Sigma) was dissolved in sodium phosphate buffer with Triton X-100 to get 60-70 units/mL of the lipase activity. One ml reaction mixture contained 99mM sodium phosphate, 149 mM sodium chloride, 0.5%(v/v) Triton X-100, 0.50 mM *p*-nitrophenylpalmitate (*p*NPP), 6-7 units porcine pancreatic lipase and 0.20 mg/mL of *Punica granatum / Nigella sativa* lipase inhibitor. The reaction mixture was incubated at 37°C for 20 min. Ethanol was added to stop the reaction. The absorbance of released *p*-nitrophenol was measured at 410 nm using a microplate reader (Varioskan Flash). The assay was performed in triplicates and the results were expressed as average value. Controls without the inhibitor were also run in parallel. Percentage inhibition of lipase activity was calculated using the formula:

$$\% \text{ Inhibition} = \frac{(\text{control-test})}{\text{control}} \text{ X } 100 \quad (1)$$

2.5.2 *In Vitro* Hormone-Sensitive Lipase (Hsl) Inhibition Enzyme Assay

The residual HSL activity for each of the two purified plant lipase inhibitors was determined as per the standard lipase inhibition assay protocol mentioned above. Hormone sensitive lipase was procured from Cayman Chemicals (human recombinant enzyme). One ml reaction mixture contained 0.50 mM *p*-nitrophenylpalmitate (*p*NPP), 6-7 units' hormone sensitive lipase and 0.20 mg/mL of purified plant lipase inhibitors.

2.6 Enzyme Inhibition Kinetics

Single phase kinetics was determined to study the inhibition kinetics. Initial rate of enzymatic reaction was found using increasing enzyme concentrations (0- 0.1mg/mL). Inhibitory kinetics was performed by maintaining fixed enzyme concentration 0.01mg/ mL

and varying substrate concentration (0-0.2mM pNPP concentration). The kinetic graph was drawn using standard Micheles-Menten curve for porcine pancreatic lipase. The double reciprocal plot was used to obtain the Lineweaver-Burk (LB) plot. Using the LB kinetic plot as reference, inhibitory assay was done with two different concentrations of the lipase inhibitor (50µg/ml, 100µg/ml) (Shamsiya et al., 2015).

2.7 Determination of IC_{50}

IC_{50} determinations were carried out in two stages using *Punica granatum and Nigella sativa* lipase inhibitors. Experiments were carried out independently for pancreatic lipase and hormone sensitive lipase using purified lipase inhibitors. IC_{50} was calculated using a range of concentrations of the lipase inhibitors ranging from 250pg/µl to 1000µg/µl following log (dose)-response inhibition curve using the equation "log (inhibitor) vs. response" with the help of software GraphPad Prism.

2.8 Specificity of the Lipase Inhibitor from *Punica granatum* and *Nigella sativa*

The lipase inhibitory specificity was assessed using different lipases: pancreatic lipase (PL), hormone sensitive lipase (HSL) (Bustanji et al., 2010), monoacylglycerol lipase (MAGL), cholesteryl esterase. Specificity of the each lipase inhibitor was determined by independently. The fluorimetric substrate, 4-methylumbelliferyl butyrate (MUB), 6.0 mg was dissolved in 1000 µL of DMSO and further diluted fivefold 10 µL of *Punica granatum* / *Nigella sativa* inhibitor and 200 µL of 50 mM phosphate buffer solution at pH 7.4 , 50 µL of substrate solution was added in each well. The fluorescence emitted at 445 nm after excitation at 365 nm was measured instantly with the fluorescence spectrometer. Each lipase was analysed separately with and without the inhibitor. The speed of fluorescence development is directly proportional to the product formation and confirms the lipase inhibitory activity (Andlauer, Prunier & Prim, 2009). Pancreatic lipase, Monoacylglycerol lipase, Hormone sensitive lipase and cholesteryl esterase were used at a concentration of 0.20µg/ml.

3. Results and Discussions

3.1 Purification of the Lipase Inhibitor from *Punica granatum* and *Nigella sativa*

The inhibitory effect of the different extracts against pancreatic lipase was determined using the standard lipase inhibitory assay. Ethanol and ethyl acetate extracts of *Punica granatum* exhibited good lipase inhibitory activity of 84.21% and 87.96% respectively and ethanol extract of *Nigella sativa* showed the highest lipase inhibitory activity of 84.93%. The HPLC fractionation of ethanol extract of both plant extracts gave good separation of the peaks (data not shown). The peak showing the highest lipase inhibitory activity was selected for further purification by preparatory TLC. Purification by neutral lipid preparative TLC resulted in the separation of the preparatory HPLC selected peak into 3 distinct bands corresponding to triglycerides, free fatty acids, diglycerides. The individual bands purified by preparative TLC were resuspended in DMSO and assessed for lipase inhibitory activity. The free fatty acids of *Nigella sativa* and triglycerides of *Punica granatum* gave the highest lipase inhibition of 92.10% and 89.65% respectively.

These purified lipase inhibitors from *Punica granatum* and *Nigella sativa* existed as mixtures of triglycerides and free fatty acids respectively and exhibited highest lipase inhibitory activity.

3.2 Structural Elucidation of the Lipase Inhibitor from *Punica Granatum* and *Nigella Sativa*

3.2.1 NMR

The ^{1}H NMR spectra of *Punica granatum* and *Nigella sativa* lipase inhibitor displayed characteristic peaks related to polyunsaturated and saturated fatty acids and no peaks were seen in the aromatic region, phenolic region or in the carbohydrate region, clearly indicating absence aromatic group, poly phenols, sugar residues.^{13}C NMR spectrum of *Punica granatum* seed lipase inhibitor and *Nigella sativa* seed lipase inhibitor confirms the presence of major unsaturated fatty acid components.

Pomegranate seed extract is mixture of triglycerides with the combination of various fatty acids in the sequence Oleic-Linoleic-Linoleic, Palmitic-Linoleic-Linoleic, Oleic-Oleic-Linoleic, Palmitic-Oleic-Linoleic and Punicic-Punicic-Punicic acid (Louise et al., 2014). The ^{1}H NMR spectrum of pomegranate oil sample revealed olefinic protons in the range of 5.30-5.45 ppm. The methine proton at position 2 of glycerol backbone was seen at 5.25-5.30 ppm

and the signals from methylene protons at position 1 & 3 are seen at 4.31 ppm & 4.16 ppm as double doublet. The methylene alpha to olefinic moiety is seen at 2.78 ppm as a characteristic triplet with *J* value of 6.6 Hz. The methylene protons alpha to carbonyl appears in the range 2.29-2.36 ppm. The methylene protons at alpha to terminal methyl appeared in the range of 1.99-2.09 ppm. The methylene protons beta to carbonyl groups appeared as multiplet in the range of 1.56 - 1.69 ppm. Also found long chain methylene protons in the range of 1.23 - 1.40 ppm as bunch of multiplet. The terminal methyl group protons appeared at 0.86 - 0.94 ppm. The ^{13}C NMR spectrum comprises of characteristic ester carboxyl signals at 178.92, 172.93 and 172.51 ppm. The intensity of former signal is double the intensity of later, a feature commonly noticed in the ^{13}C NMR spectrum of TAG. Bunch of olefinic carbon signals were seen between 127 - 130 ppm. These signals correlate with olefinic protons at 5.30 - 5.45 ppm in 2D HSQC spectrum. The methine carbon of glycerol backbone appears at 68.58 ppm and the two equivalent methylene carbon signals appear together at 61.78 ppm. These signals correlated with methine at 5.25-5.30 ppm and methylene protons from glycerol back bone at 4.31 ppm & 4.16 ppm respectively in 2D HSQC spectrum. All the methylene carbon signals appeared between 22 ppm to 33 ppm was further confirmed by spin echo Fourier transform spectrum which assorts methylene signals in negative phase. The terminal methyl carbon signals appeared at 13.76 ppm and 13.79 ppm. As the pomegranate seed extract was a mixture of various triglycerides, the equivalent protons and carbon signals appeared collectively in terms of chemical shift values and integration values.

The nigella seed extract is a mixture of free fatty acids like C16: 0 (palmitic acid) ,C18:2 (linoleic acid), C18:1 (oleic acid), C18:3 (linolenic acid) and a small fraction of corresponding methyl esters. This was confirmed by GC-MS. The ^{1}H and ^{13}C NMR spectrum of nigella seed extract was also recorded. The distinct feature noticed with nigella seed extract as compared to pomegranate seed extract was the absence of TAG as inferred by the absence of glycerol backbone. ^{1}H NMR spectrum of nigella seed extract includes olefinic protons in the range of 5.24 - 5.46 ppm. The ester methyl proton signal was seen at 3.65 ppm as a singlet. The methylene protons alpha to olefinic system from linoleic and linolenic acid were seen at 2.8 ppm as a triplet with *J* value of 6.4 Hz. The methylene protons alpha to carbonyl appears in the range 2.24 - 2.39 ppm. The methylene protons at alpha to terminal methyl appeared in the range of 2.01 - 2.12 ppm. The methylene protons beta to carbonyl groups appeared as multiplet in the range of 1.56 - 1.69 ppm. The long chain hydrocarbon

protons in the range of 1.29 - 1.45 ppm as multiplet. The terminal methyl group protons appeared at 0.86 - 0.97 ppm. The ^{13}C NMR spectrum consists of carbonyl signals from fatty acid and methyl esters in the range of 173 - 176 ppm. The olefinic carbon signals appeared between 127 and 129 ppm. The methylene carbon signals appeared between 21-34 ppm range. Among these 22.1 ppm & 21.21 ppm methylene carbon signals were alpha to terminal methyl and 31.11 ppm & 31.51 ppm signal were beta to terminal methyl as identified by HMBC correlations. The terminal methyl signals from a mixture of fatty acid appeared collectively at 13 ppm. The correlation between ^{1}H and ^{13}C nuclei was studied by 2D HSQC experiment. Here again the as the nigella seed extract was a mixture of various fatty acids the equivalent protons and carbon signals appeared collectively in terms of chemical shift values and integration values.

3.2.2 Determination of Triglyceride Composition *of Punica Granatum* Lipase Inhibitor and Fatty Acid Composition of *Nigella Sativa* Lipase Inhibitor Using Gc -Ms

The GC–MS evaluation of fatty acid isolate of *Nigella sativa* lipase inhibitor showed that, the lipase inhibitor comprised of 3 unsaturated fatty acids and one saturated fatty acid, with C18:2 linolenic acid being the major one, comprising of about 56.6 % of the total area percentage. GC-MS analysis of *Punica granatum* lipase inhibitor resolved into 3 fatty acids containing large amounts of C18:3 fatty acids (86.76 % of total area percentage). This inhibitor appeared to be a mixture of triglycerides, with the high probability of one of the triglyceride being made of 3 Punicic acids (Louise et al., 2014), (Topkafa, & Sherazi, 2015). The other triglycerides appeared to have different combinations of C16 : 0, C18 : 2 and C18 : 3. Louise et al, reported that the most commonly occurring triglyceride orientations in pomegranate seed oil extract are Palmitic-Linoleic-Linoleic TAG, Punicic-Punicic-Punicic TAG, Oleic-Oleic-Linoleic TAG, Palmitic-Oleic-Linoleic TAG, Oleic-Linoleic-Linoleic TAG (Louise et al., 2014). In our studies, GC-MS analysis indicated that the most probable TAGs present in SHAMstat3pg are Punicic-Punicic-Punicic TAG, Oleic-Oleic-Linoleic TAG and Oleic-Linoleic-Linoleic TAG (Fig. 1). Triglycerides with fatty acid compositions other than oleic acid, linoleic acid and punicic acid could be ruled out as they were not detected. All possible TAGs present would have oleic acid, linoleic acid and punicic acid. GC –MS analysis of *Nigella sativa* lipase inhibitor confirmed the presence of 3 unsaturated fatty acids and one saturated fatty acid as a mixture of free fatty acids (Fig. 2). The predominant fatty

acid being linolenic acid and linoleic acid. These fatty acids work in a synergistic way to work as a functional lipase inhibitor.

The triglyceride cocktail from *Punica granatum* was named SHAMstat3pg and fatty acid cocktail from *Nigella sativa* was called RAYstat4ns. These nominations will be used in our further discussions.

Table 1 *Fatty acid composition of Nigella sativa lipase inhibitor RAYstat4ns and Punica granatum lipase inhibitor SHAMstat3pg*

Fatty acid constituent of	**Area% fatty acid of** RAYstat4ns	**Area% fatty acid of** SHAMstat3pg
C16 : 0 (palmitic)	10.59	-
C18 : 1 (oleic)	4.97	2.88
C18 : 2 (linoleic)	27.80	10.36
C18:3 Punicic Acid(CLA)	-	86.76
C18:3 (linolenic)	56.66	-

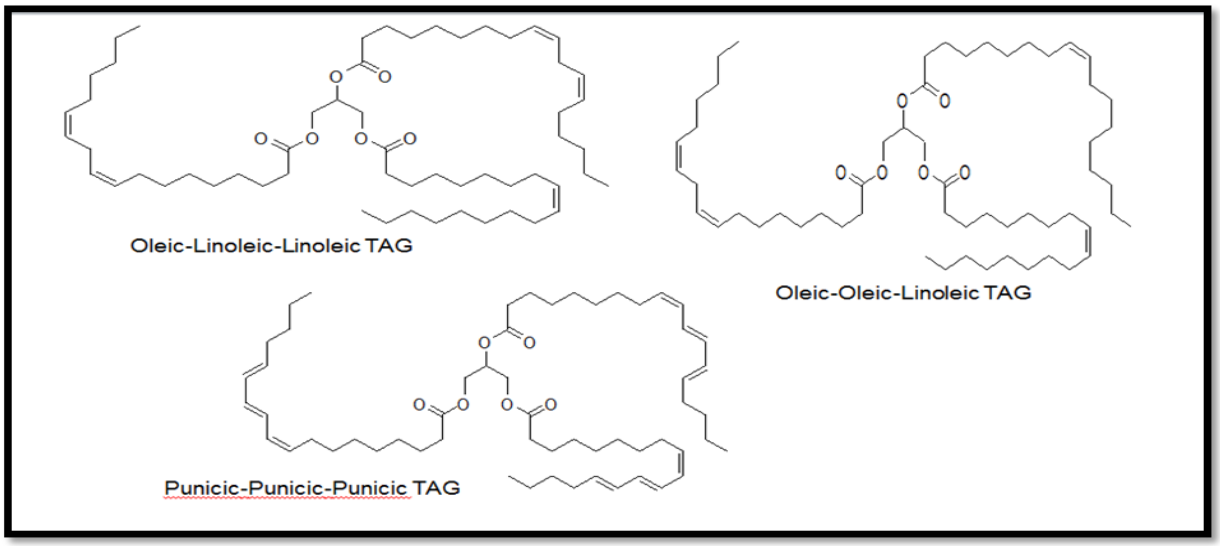


Figure1: *Triglyceride composition of Punica granatum lipase inhibitor SHAMstat3pg*

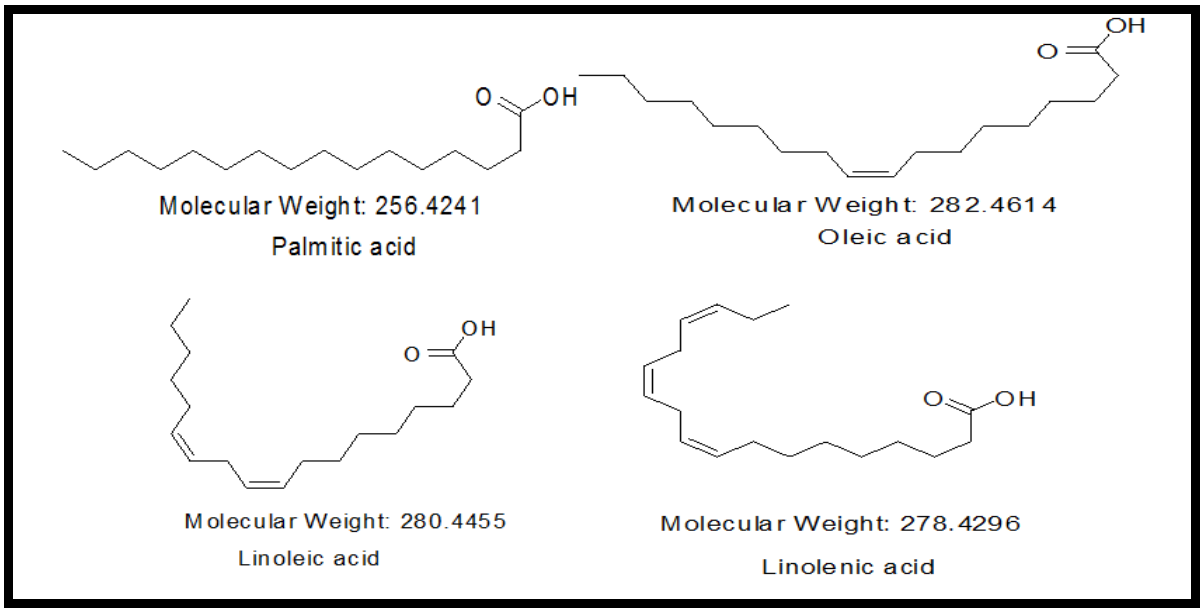


Figure 2: *Fatty acid composition of Nigella sativa lipase inhibitor RAYstat4ns*

3.3 Kinetics of Inhibition

The kinetic properties of purified inhibitor RAYstat4ns and SHAMstat3pg was studied using different substrate concentrations ranging between 0-0.2mM. These lipase inhibitors showed mixed type of inhibition (Fig.3, Fig.4). The apparent V_{max} was observed to change, because the inhibitor is capable of preventing catalysis regardless of whether the substrate is bound to the enzyme or not. Also, inhibitor site is remote from the active site. These mixed type inhibitors, inhibit the enzyme activity by causing a conformational change which prevents enzyme from converting substrate to product (Villa-Ruano, Zurita-Vásquez, Pacheco-Hernández, Betancourt-Jiménez, Cruz-Durán, & Duque-Bautista, 2013). The structure function relationship between the enzyme and RAYstat4ns and SHAMstat3pg could help us understand the pharmacophore for pancreatic lipase and hormone sensitive lipase inhibition in order to treat obesity .

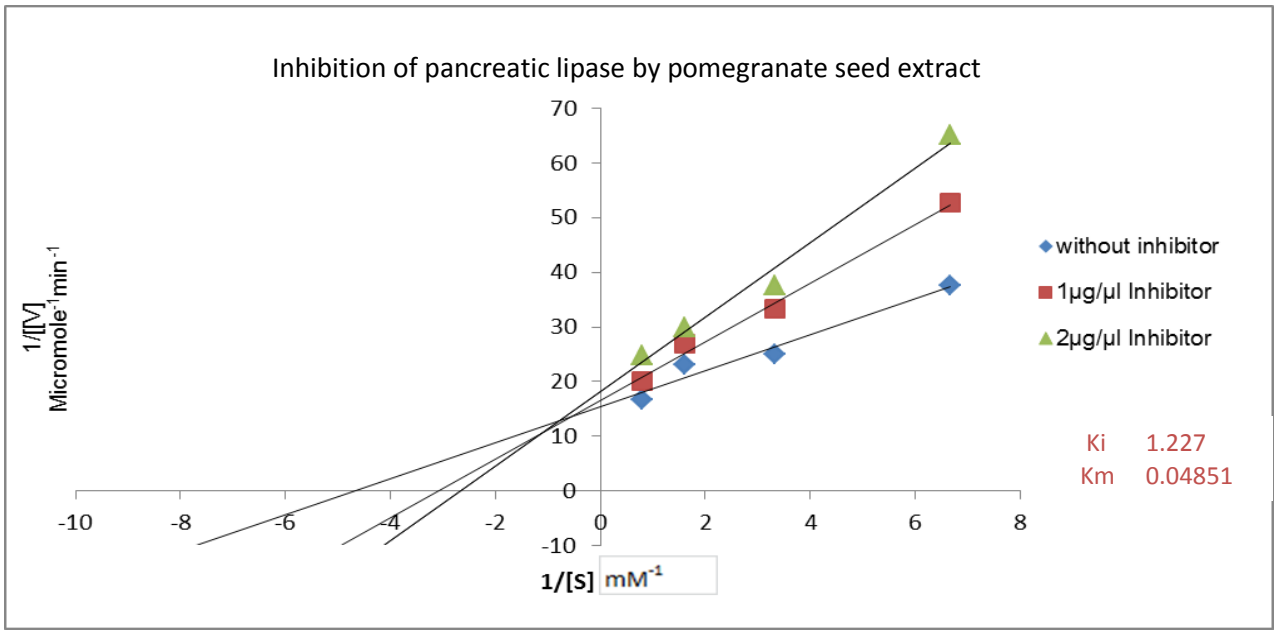
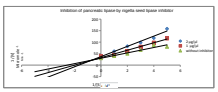


Figure 3: *Inhibition kinetics pomegranate seed extract*



Ki 1.290
Km 0.04132

Figure 4: *Inhibition Kinetics Nigella Seed Extract*

3.4 Determination of IC_{50} of RAYstat4ns and SHAMstat3pg

The experimental IC_{50} of the lipase inhibitors, RAYstat4ns and SHAMstat3pg was found to be 4.02µg/µl and 7.35 µg/µl for pancreatic lipase respectively and 6.31 µg/µl and 11.45 µg/µl respectively for hormone sensitive lipase (Table 2). However, the partially purified (preparatory HPLC purified) lipase inhibitors of *Nigella sativa* and *Punica granatum* showed an IC_{50} of 4µg/µl and 10 µg/µl against pancreatic lipase respectively and an IC_{50} 15 µg/µl and 18 µg/µl against hormone sensitive lipase respectively. There is a considerable increase in the sensitivity in terms of IC_{50} values during purification of the lipase inhibitors.

This was compared with IC_{50} of Orlistat (commercial inhibitor, sigma) against pancreatic lipase and hormone sensitive lipase, which was respectively 0.76 µg/µl and 3.3 µg/µl. *Nigella sativa* lipase inhibitor RAYstat4ns showed better inhibition than *Punica granatum* lipase inhibitor for both pancreatic and hormone sensitive lipase. However, Orlistat exhibited better inhibition compared to both RAYstat4ns and SHAMstat3pg.

Table 2: *IC_{50} Values (µg/ml) of Nigella and Pomegranate Lipase Inhibitors*

Inhibitor	Inhibition (IC_{50}) of	
	Hormone sensitive lipase (HSL) µg/µl	Pancreatic Lipase(PL) µg/µl
HPLC purified *Punica granatum* lipase inhibitor	15	4
HPLC purified *Nigella sativa* lipase inhibitor	18	10
RAYstat4ns	6.31	4.02
SHAMstat3pg	11.45	7.35
Orlistat	2.3	0.76

3.5 Specificity of Raystat4ns and Shamstat3pg

The fluorimetric assay which is more sensitive than the colorimetric lipase inhibition assay was used to study the specificity of the plant lipase inhibitors. Four different lipases, pancreatic lipase (PL) and hormone sensitive lipase (HSL), monoacyl glycerol lipase (MAGL) and cholesteryl esterase were used as targets for the inhibitor in four independent experiments. Among the four different lipases used, the lipase inhibitors showed higher inhibition with pancreatic lipase (PL) and hormone sensitive lipase (HSL). The inhibiton by RAYstat4ns was only 15.54% towards monoacyl glycerol lipase (MAGL) and inhibition of MAGL by SHAMstat3pg was 25.65% These inhibitors did not show any significant inhibition against cholesteryl esterase as well (Fig 5).

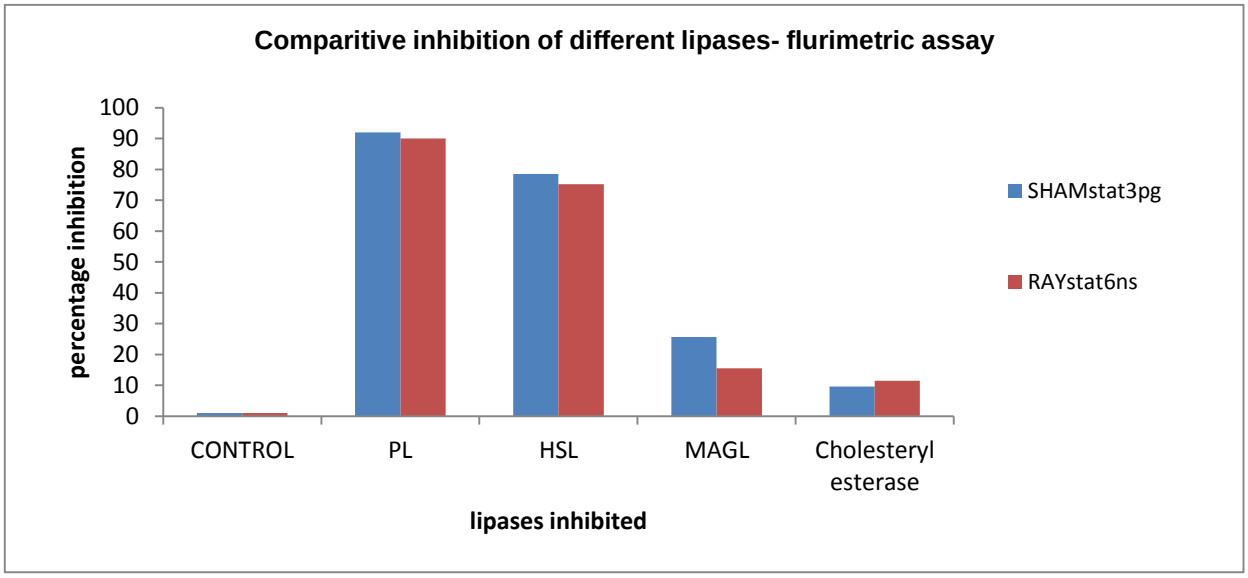


Figure 5: *Comparative inhibition of different lipases PL, HSL, MAGL, Cholestery esterase by the inhibitor using different assays*

Thus, lipase inhibtors RAYstat4ns and SHAMstat3pg from *Nigella sativa* and Punica *granatum* seeds respectively are a cocktail of fatty acids existing as such or as triglycerides. These have been purified by preparative HPLC and TLC. RAYstat4ns is a mixture of free fatty acids and SHAMstat3pg, a mixture of triglycerides. The composition has been studied in detail using NMR and GC-MS. There were no interfering volatiles and phenolics in the purified lipase inhibitors.

4. Conclusion

RAYstat4ns and SHAMstat3pg are a mixture of fatty acids and triglycerides respectively. RAYstat4ns is made up of C16: 0 (palmitic acid), C18: 1 (oleic acid), C18: 2 (linoleic acid), C18:3 (linolenic acid), one saturated fatty acid and five unsaturated fatty acids. SHAMstat3pg exists as a cocktail of triglycerides made up of three unsaturated fatty acids, C18: 1 (oleic acid), C18: 2 (linoleic acid), C18:3 Punicic Acid (CLA). Since RAYstat4ns and SHAMstat3pg have shown extremely good inhibitory activity against pancreatic lipase and hormone sensitive lipase with a good $IC_{50.}$ These inhibitors could be used as a treatment for obesity as well as for prevention of type 2 diabetes and insulin resistance. These isolated lipase inhibitors also carry with them all the proven benefits of dietary fatty acids, saturated and poly unsaturated. Hence, making dietary fatty acids significant and efficient therapeutic molecules. To our knowledge, there are no approved

plant based lipase inhibitors that have been tested *in vivo*. RAYstat4ns and SHAMstat3pg could prove to be two such simple, safe and efficient plant based lipase inhibitors with multifaceted bioactivities. Further study is required to understand the mode of action of RAYstat4ns and SHAMstat3pg and other dietary fatty acids as lipase inhibitors to combat obesity and prevent insulin resistance. The role of all the individual fatty acids in RAYstat4ns and SHAMstat3pg could be mapped out to clearly understand the contributing activities of the respective fatty acids in preventing obesity. There is immense potential for research in exploiting plant based dietary fatty acids as therapeutic agents for treatment of obesity and prevention of type 2 diabetes. RAYstat4ns and SHAMstat3pg, plant based lipase inhibitors with forward study could prove to be competent nutraceuticals for treatment of obesity.

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