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Original article

Antilipase activity guided fractionation of *Vinca major*

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ABSTRACT

Methanolic extract of flowers of *Vinca major* (VFE) reduced the activity of porcine pancreatic lipase (PPL) which was reported in our previous study. To identify the antilipase molecule(s) in the VFE, the antilipase activity-guided fractionation for the purification of lipase inhibitory molecule(s) by Silica Gel and Sephadex G-50 column chromatography was attempted, and the final anti-lipase fraction was examined by ESI-MS and FT-IR spectroscopy. Two molecules namely, Majoridine and Akuammine were elucidated to be present in the flowers of *V. major* which possessed high PPL inhibition activity. Further, both molecules were looked *in silico* for their interactions with PPL and other important proteins of lipid metabolism using molecular docking studies. Majoridine and Akuammine demonstrated significant binding interactions with active site residues of PPL.

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1. Introduction

Obesity or adiposity, the 'New World Syndrome' has now been declared as a serious health concern in both developed and developing nations. It is one of the metabolic disorders which increases with age and reduce a person's life anticipation by 5–15 years (Montesi et al., 2016). It is linked with serious clinical outcomes, *viz*. stroke, sleep apnea, osteoarthritis, cardiovascular problems and type-2 diabetes (Cliff et al., 2016). It is also tied up with some forms of cancer, periodontal diseases and pneumonia (Cabia et al., 2016). There were over 600 million adults worldwide as clinically obese and 1.9 billion as overweight up to 2014 (World Health Organization, 2016). In the European Union, 60% of adults and 20% of school-age children have been tagged as overweight or obese (Baker and Bate, 2016). In England, 24% of adult population is obese and further 36% is overweight (Public Health England, 2016). In USA, 13 million children (aged 2–19) and 79 million adults (aged 20 and older) have been declared obese by American Heart Association Statistics Committee (Skinner et al., 2016). In Australia, 80% adults have been predicted to be obese up to 2025 (Australian National Preventive Health Agency, 2014). Obesity figures have been demonstrated in Indian population too (Ranjani et al., 2016).

High energy diets and reduced physical movement are the major causes of development of this disorder. Obesity can be diagnosed by body mass index (BMI), weight distribution, waist circumference and associated comorbidities (Singh et al., 2013). A BMI of 30 or more indicates the person as obese or a person with waist circumference 40 (for man) and 35 (for woman) inches considered as obese. Medications for obesity and associated comorbidities are available in the market but they have intolerable side effects. Currently approved medicines (Orlistat, Phentermine and Sibutramine) lose only 5–10% weight (at 1 year use) which is very far from the cure of this epidemic. Additionally, patient regains the weight over a period of time. Therefore, prevention of this epidemic has become a major focus of research after cancer epidemic in the developed nations at present (Barnes, 2015; Pandita et al., 2016). Diminution of nutrient digestion and absorption through lowering the activity of enzymes involved in food digestion is the trending strategy for the same (Martin et al., 2015). Major components which contribute to obesity/adiposity are dietary fats, and the major responsible enzyme for the digestion of these fats is pancreatic lipase (PL). Consequently, inhibition or reduction of this enzyme will lead to the reduced digestion of dietary fats and concomitant absorption. Current clinically recommended pancreatic lipase inhibitor 'Orlistat' reduces the body fat (up to 30% during 1 year of use) with side effects *i.e.* fatty/oily spotting, gastric irradiations, stomach pain, increased bowel movements, reduced vitamin absorption, drowsiness, short of breath *etc*. Therefore, a safe pancreatic lipase inhibitor is still highly sought therapeutic molecule in the drug/pharmaceutical market, and plant derived natural

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products are suited option for the same. Several plants have been reported in literature for their lipase inhibitory effects, *viz*. *Platycodon grandiflorus* (Zhao et al., 2005), *Cyclocarya paliurus* (Kurihara et al., 2003), *Dioscorea nipponica* (Kwon et al., 2003), *Scabiosa tschiliensis* (Zheng and Koike, 2004), *Panax japonicas* (Han et al., 2005), *Aesculus turbinate* (Kimura et al., 2006), *Acanthopanax sessiliflorus* (Yoshizumi et al., 2008), *Glycyrrhiza auralensis* (Won et al., 2007), *Peganum harmala*, *Inonotus hispidus* (Benarous et al., 2015), *Calatropis procera* (Patil et al., 2015), *Malus domestica*, *Vitaceae vitis* (Danis et al., 2015) *etc*.

Our laboratory is engaged in discovering the lipase inhibitory natural products from the Western Himalayan flora (Shimla, India). Interestingly, the methanolic extract of flowers of *Vinca major* (VFE) has the potential to reduce the activity of porcine pancreatic lipase (Singh et al., 2014). *Vinca major* (the big leaf periwinkle) is an evergreen shrub and belongs to the *Apocynaceae* family which is very well known for its alkaloids. The plants of *Vinca* genus have been reported to possess medicinal properties against hypertension, leukemia and cancer. *Vinca major* is native to Mediterranean Europe, Asia Minor and Northern Africa. This plant bears flowers from mid January to mid March in Shimla, India. It spreads vegetatively by arching stolons which roots at tips. It has creepy non-flowering stem and erect flowering stem up to 2 ft. Leaves are glossy, 2–3 in number (4–7 cm long), caudate at base with ciliated hairy margins. All these characteristics are based on the observation recorded for *Vinca major* plants existing in Shimla and its surroundings. In the continuing work we evaluated the PPL inhibition kinetics of VFE and fractionated it on gel filtration matrices (Silica Gel and Sephadex-G-50) for the identification of lipase inhibitory molecule(s).

2. Materials and methods

2.1. Plant material and chemicals

Aerial parts of *Vinca major* were collected from forest areas of district Shimla (India), *viz*. Summer Hill, Potter Hill, Saangti, Chailii, Andari and Taradevi located at 31.61°N 77.10°E. Taxonomic identity of *Vinca major* was confirmed by Herbarium Staff, Systematic Botany Discipline, Forest Research Institute, Dehradun- 248 006, India. Porcine pancreatic lipase (PPL) and Steapsin (SRL, Mumbai, India); Lipolase100L (Novozymes, Bangalore, India); O'Stat (Orlistat; ARISTO Pharmaceuticals, Mumbai, India) and *p*-Nitrophenyl palmitate (*p*-NPP; Lancaster Synthesis, England) were procured from various commercial suppliers. All other chemicals were of analytical grade and were used as received.

2.2. Measurement of PPL inhibitory activity

Various amounts of plant extract or solvent fractions were pre-incubated with 20 μl of PPL (36–44 U/ml) at 37 °C for 20 min in 1X phosphate buffered saline (PBS; pH 7.2) with reaction volume 2.96 ml. Then 40 μl of *p*-NPP (10 mM in *iso*-propanol) was added as substrate in a final volume of 3.0 ml. This reaction mixture was incubated at 37 °C for 5 min and reaction was terminated by incubating at −20 °C for 10 min. Amount of *p*-nitrophenol released was measured at 405 nm wavelength in a UV–Visible spectrophotometer (3000+, Lab India, Mumbai). Assays were performed in duplicate and mean values were presented in each experiment. Initial and residual lipase activities of PPL preparation were recorded and percent PPL inhibition (if any) was calculated.

2.3. Determination of kinetic behaviour of lipase inhibition by VFE

PPL was assayed with increasing concentrations (2.5, 5, 10 and 20 mM) of *p*-NPP in the absence and presence of three concentrations (0.20, 0.25 and 0.30 mg/ml) of VFE. Inhibition mode for PPL activity was determined by the V_{max} and K_m values obtained from the Michaelis–Menten kinetics of VFE at the mentioned concentrations.

2.4. Antilipase activity guided fractionation of VFE

Shade dried flowers (5 g) of *Vinca major* were grounded, suspended in 100 ml petroleum ether and extracted overnight. Next day, petroleum ether layer was collected and evaporated at 37 °C to yield the ether fraction (31 mg; 0.62% percent extractive). Remaining residue was then extracted overnight with 100 ml benzene and fraction was collected by evaporation (34 mg; 0.68% percent extractive). Likewise, chloroform, acetone, methanol and water extracts were also obtained. The process resulted in completely dried 59 mg chloroform extract (1.18% percent extractive), 42 mg acetone extract (0.84% percent extractive), 88 mg methanol extract (1.76% percent extractive) and 107 mg aqueous extract (2.14%) from the *V. major* flowers. Each of the solvent extract was tested for PPL inhibition using same procedure as described above. In brief, 200 μl of each of the solvent extracts (1 mg/ml stock in 0.05 M PBS, pH 7.2) was pre-incubated with 20 μl PPL (36–44 U/ml) at 37 °C for 20 min in 1X PBS (pH 7.2). Reaction was then started by adding *p*-NPP (40 μl; 10 mM) in a final reaction volume of 3.0 ml. After 5 min incubation at 37 °C, A_{405} values were recorded, residual PPL enzyme activities calculated and percent PPL inhibition were calculated. Fractionation of the active methanolic extract was performed by Silica gel and Sephadex G-50 column chromatography. In brief, 4 g of silica gel (particle size 0.040–0.063 mm) was suspended in 20 ml *n*-hexanol and packed (wet packing) in a sintered glass column (15.4 cm length × 1.5 cm internal diameter). 2 ml of methanolic extract (stock 1 mg/ml) was loaded atop the column. Column was then subjected to elution using selected alcohols of increasing polarity with order being, *n*-octanol < *n*-hexanol < *n*-pentanol < *n*-butanol < *n*-propanol < ethanol < methanol. The aqueous mobile phases (1: 1 ratio; solvent: water) were used with octanol, hexanol and pentanol fractionation which didn't affect the chromatography. Ten eluted fractions (each of 0.6 ml) with each alcohol were collected and stored at −20 °C for PPL inhibitory assays. Each of these alcohol fractions (200 μl) were pre-incubated with PPL for 20 min followed by addition of substrate *p*-NPP, and residual activities were recorded. The solvents alone were not observed to affect the activity of commercial PPL. Among the silica fractions, *n*-butanol fraction (fraction ID B_8) showed maximum PPL inhibition (41%), and this fraction was also examined by spotting it on a preparative TLC plate coated with silica gel 60 F_{254} (Aluchro Sep Silica Gel 60, 50 mm × 100 mm × 0.2 mm). In brief, B_8 fraction was spotted on the baseline drawn about 1 cm from the bottom of the TLC sheet by using 0.5 μl micropipette tip. Spots were dried and TLC plates were developed in a chromatographic tank saturated with vapors of the mobile phase (1:1 *n*-hexanol: *n*-methanol) at room temperature (average 28 °C). Compounds present in B_8 fraction were detected by spraying 0.25% vanillin-sulfuric acid solution. The vanillin-sulfuric acid reagent detects natural compounds such as higher alcohols, phenols, steroids, flavonoids and essential oils. The colouring reagent was prepared by dissolving 0.25 g of vanillin crystals in sulfuric acid followed by addition of ethanol. Developed TLC plates were sprayed with this solution and heated at 65 °C in oven for 10 min. A smear was obtained in B_8 fraction. The B_8 fraction was further subjected to Sephadex G-50 column chromatography. In brief, 2.5 g of Sephadex G-50 was suspended in 25 ml of water, boiled in microwave oven, and left to stand for 2 h prior to use. The slurry was packed in a sintered glass column (length 16.8 cm × 1.5 cm internal diameter; Vt 29.7 cm^3), allowed to settle and equilibrated with *sec*-Butyl alcohol. The 2 ml of above butano-

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