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Inhibitory effect of Persea americana Mill leaf aqueous extract and its fractions on PTP1B as therapeutic target for type 2 diabetes

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Abstract: Persea Americana Mill. (Lauraceae), is a popular plant in Cuba due to its nutritional and medicinal properties. The fruit of the plant is commonly known as avocado. The leaves of Persea americana Mill. have been popularly used in the treatment of diabetes in countries in Latin America and Africa. The present study is aimed to explore one of the underlying mechanisms that mediate the antidiabetic efficacy of Persea americana Mill. The aqueous extract from the leaves of the plant and its fractions were evaluated on the inhibitory activity of the protein tyrosine phosphatase 1B (PTP1B) as target of type 2 diabetes. The results revealed that aqueous extract from P. americana inhibited the enzymatic activity of PTP1B in an extract concentration dependent manner, resulting mainly active the most polar fraction. The present research demonstrated that aqueous extract from P. americana and polar fraction (PaF10) have promissory antidiabetic properties mediated by PTP1B, which is a relevant mechanism involved on insulin resistance in type 2 diabetes.

Keywords: Persea americana Mill., antidiabetic activity, PTP1B inhibitory activity.
ABREVIATIONS LIST:
PTP1B- protein tyrosine phosphatase 1B-

INTRODUCTION
Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia and altered metabolism of lipids, carbohydrates and proteins, due to defects in insulin secretion and/or insulin action (ADA, 2012). Protein tyrosine phosphatase 1B (PTP1B) has been implicated in the negative regulation of insulin signaling by dephosphorylating the insulin receptor (IR) as well as its substrate, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), and selective inhibition of PTP1B also has the potential to cause weight loss, which is a benefit because obesity is an important component of the type 2 diabetic pathology (Delibegović and Mody, 2009).

Persea americana Mill. (Lauraceae), is one of the popular Cuban plant which is recognized by its nutritional and medicinal properties. The fruit of the plant is commonly known as avocado and constitutes the proper part used as food. The root, bark, fruit, and leaf are used extensively in traditional medicine in many tropical and subtropical countries for the treatment of various ailments (Mbang et al, 2005). In Nigeria, several ethnic groups use the leaves of P americana in the treatment of hypertension (Mbang et al, 2005). Cuban ethnomedicine, report some medicinal uses of the leaves as well: antitussive, carminative, anti-diarrhea, influenza, abortifacient, diuretic, cholagogue, depurative, spasmogenic, musculotropa, stomachic; indicated in cases of amenorrhea, liver obstructions and excess uric acid (Roig, 1988). The leaves of Persea americana Mill. have been popularly used in the treatment of diabetes in countries in Latin America and Africa (Lima, 2012). The aim of the study was to investigate the inhibitory effect on PTP1B of Persea americana Mill. leaves aqueous extract and its fractions as the underlying mechanism related with the antidiabetic potentiality of the plant, for type 2 diabetes.

MATERIALS AND METHODS
Plant material
Persea Americana Mill. (Lauraceae) leaves were collected in San José de Las Lajas, Mayabeque, Cuba. Plants were taxonomically authenticated by Prof. Fernando Franco Flores, in the Faculty of Agronomy at the Agriculture University of Havana, Cuba and a voucher specimen of the plant is stored for reference (Nº 01-2013) in the Herbarium of this institution for further reference. Fresh leaves of P americana were dried in a lab stove at 37° C during 96 h. The dry leaves were milled in fine particles of 5 mm. The plant extracts were obtained from powder aqueous extraction at 95° C for 30 min, in a 10% (w/v) relation. The resulting extracts were freeze dried. Dry extracts were reserved in suitably plastic bottles and stored in desiccators for future experiments.

Plant aqueous extracts fractioning
Ten fractions were obtained from P americana aqueous extract using Flash Chromatography System. It was used 500 mg of dried extract in an adsorption chromatography employing an analytical column (Isolute Flash, SI, 20g, Biotage) and as mobile phase, an organic solvents mixture, from low to high polarity (n-hexane; 1,2 di-chloro-metane; 2-butanol; methanol and water) purchased from Sigma-Aldrich. Elution was achieved using solvent gradients of 400 ml aliquots of n-hexane 100% (PaF1, 0.5 mg); 1,2 di-chloro-metane 100% (PaF2, 2.7 mg); 1,2 di-chloro-metane 75%: 2-butanol 25% (PaF3, 2.3 mg), 1,2 di-chloro-metane 50%: 2-butanol 50% (PaF4, 1.3 mg), 1,2 di-chloro-metane 25%: 2-butanol 75% (PaF5, 1.4 mg), 2-butanol 100% (PaF6, 2.3 mg), 2-butanol 75%: methanol 25% (PaF7, 13.5 mg), 2-butanol 50%: methanol 50% (PaF8, 81.6 mg), 2-butanol 25%: methanol 75% (PaF9, 52.5 mg) and methanol 50%: water 50% (PaF10, 88.6 mg).

Protein tyrosine phosphatase 1B (PTP1B) enzyme assay.
The assay was achieved as previously was described (Montalibet et al., 2005). The experiments were performed on 96 micro well plates. PTP1B (human, recombinant) was purchased from Sigma. For inhibition assay 25 µl of different concentrations of P americana aqueous extracts and its fractions (0.01-300 µg/mL) were evaluated. The standard inhibitor [Bis(4- Trifluoromethylsulfonamidophenyl)-1,4-diisopropylbenzine] (TFMS, Calbiochem) (0.1-300 µM) or buffer solution (pH = 7.2), containing the following reactives from Sigma at final concentrations indicated: Hepes (25 mM), sodium chloride (50 mM), Dithiothreitol (2 mM), ethylene-diamine-tetraacetic acid (EDTA) (2.5 mM) and Bovine Serum Albumin (BSA) (0.01 mg/ml), were preincubated with 50 µl of enzyme for
30 min at 37°C. After 25µl of substrate (6, 8-difluoro-4-methylumbelliferyl phosphate) (DiFMUP, Invitrogen), at final concentration of 10 µM, was added to reaction mixture and it was placed in a 37°C incubator for 10 min. The amount of end product obtained was estimated by measuring the fluorescence changes using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Statistical analysis
The results were analysed using the Software GraphPad Prism version 4.03, 2005 (La Jolla, California, USA). Data expressed as mean ± SEM for 4 replicates of the experiments.

RESULTS
Effect of Persea americana Mill. aqueous extract on PTP1B enzymatic activity.
P. americana aqueous extract inhibited the enzymatic activity of PTP1B in an extract concentration dependent manner, reaching inhibitory values near to 100 % to a concentration of 100 µg/ml. The Ki was 1.2 ± 0.15 µg/ml (Figure 1).

Effect of Persea americana Mill. fractions obtained from aqueous extract on PTP1B enzymatic activity.
Figure 2 shows that the fractions 1 and 2 produced a slight inhibition of PTP1B activity; while the fraction 5 exhibited a moderate inhibition of this protein. Further fractions showed a potent effect on PTP1B enzymatic activity, resulting fractions 3 and 6, the most actives in this assay. The percentage of inhibition values in decreasing order were: F6 (99.3%), F3 (98.9%), F9 (96.2%), F7 (95.5%), F10 (95.1%), F4 (89.7%), F8 (89.0%), F5 (63.4%), F2 (29.4%), F1 (16.8%).

Figure 1
Dose response curve of P americana aqueous extract effect on PTP1B enzymatic activity expressed as percentage of control (100%). Values represent mean ± SE for each concentration tested.
Figure 2
Screening of fractions at 100 µg/ml obtained from P americana aqueous extract as PTP1B inhibitors. Values are expressed as a percentage of PTP1B enzymatic activity of control (100%). Values represent mean ± SE for each fraction tested (n = 3).

Dose response curves of active fractions from aqueous extract of Persea americana Mill. on PTP1B enzymatic activity
The Figure 3 (A, B, C, D, E, F and G) shows dose-response curves of the more active fractions, obtained from the aqueous extract of P americana leaves on enzymatic activity of PTP1B. The shape of the curves are similar in all cases with a mean Hill slope value of -2.48 ± 0.53. The graphics show that PTP1B inhibitory activity increased in a dependent manner according to the concentration of the fractions, reaching inhibition values equal or close to 100% at the maximum concentration tested (300 µg/ml). Differences were found in the Ki values among different fractions. Fraction 10 showed a Ki = 1.2 ± 0.06 µg/ml, which is indicative that this fraction was the most effective in the inhibition of PTP1B activity.
DISCUSSION

The leaves of Persea americana Mill. (Lauraceae) have been popularly used in the treatment of diabetes in countries in Latin America and Africa (Lima et al., 2012). It was also reported that the aqueous leaf extract of P. americana possesses hypoglycemic effects in the normal rats. The maximum antidiabetic activity was reached at 6 h after a single dose of the extract was administered, producing 60.02 ± 6.83% reduction in blood glucose level (Anita et al., 2005).

The present study was approached to explore the possible mechanism, mediated by PTP1B enzyme inhibition, which could be underlying the P. americana antidiabetic activity observed in ethnomedicine (Lima et al., 2012). Protein tyrosine phosphatase 1B (PTP1B) has been implicated in the negative regulation of insulin signaling by dephosphorylating the insulin receptor (IR) as well as its substrate, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), so according to that selective inhibition of
PTP1B has emerged as a potential drug target for the treatment of type 2 diabetes (Teng et al., 2011).

A phytopharmacological review on Persea americana showed that avocados are a rich source of nutrients and phytochemicals as well (Yasir et al., 2010). Major chemical constituents of the different parts of the plant of P. americana Mill. has been reported (Ding et al., 2007). The compounds classes were separated into aliphatic acetogenins or alkanols (del Refugio et al., 2004), terpenoid glycosides (Rodriguez et al., 1999), various furan ring-containing derivatives (Werman and Neeman, 1990) and flavonoids (Litz et al., 2005). The highly functionalized alkanols (1-17) of avocado have exhibited quite diverse biological properties (Kawagishi et al., 2001).

In our study most PTP1B inhibitory activity was found in P. americana more polar fraction, corresponding with fraction PaF10. This fraction might probably contain tannins, although further experiments on chemical characterization of this fraction will be necessary to demonstrate it. It has been confirmed the importance of tannins in the PTP1B inhibitory effect, comparing results of total methanolic extract and detanified extract of Cichorium intybus demonstrated that tannins are responsible components of this pharmacological effect (Muthusamy et al., 2008). Other plant extracts have showed inhibitory effect on PTP1B enzymatic activity in the same range of concentrations of P. americana leaves, evaluated in the present work. Thus, the methanolic extract of Psidium guajava was reported to have a significant inhibitory effect of 87%, at a concentration of 30 μg/mL, resulting, after the extract fractionation into four sub-fractions by solvent partition, butanol fraction as the active fraction with an IC₅₀ value of 2.6 μg/ml (Oh et al., 2005). Other constituents have been also reported with that activity, for example terpenoids compounds (Yu et al., 2005; Thuong et al., 2008). Thus, it is possible that terpenoid glycosides could contribute to PTP1B inhibitory effect in the total extract and it can be presents in other evaluated fractions.

Previous studies have shown that PTP1B enzyme has two active sites: a catalytic site and an allosteric site. By X-ray diffraction have been identified the interactions between PTP1B and allosteric inhibitors (Lee et al., 2008). A general mechanism proposed for other proteins tyrosine phosphatases, is that allosteric inhibitors prevent the formation of the enzyme active form, blocking the WPD loop mobility in the catalytic site, which closure is essential for catalytic activity of the enzyme (Wiesmann et al., 2004). Hansen et al., 2005 reported that the allosteric inhibition of PTP1B activity can be achieved by selective modification of cysteine residues, Cys 121, which although is not located in the catalytic site, providing interactions with residues which are in contact with the His 214, which has been shown to be important for catalysis. Thus, the amentoflavone obtained from Selaginella tamariscina, a biflavonoide, inhibited PTP1B activity by allosteric inhibition (Lee et al., 2008).

Previous studies has confirmed that the hydroalcoholic extract of the leaves of Persea americana has anti-diabetic properties and possibly acts to regulate glucose uptake in liver and muscles by way of PKB/Akt activation, restoring the intracellular energy balances (Lima et al., 2012). Other authors evaluated the insulin-stimulative and anti-oxidative effects of Persea americana fruit extract using streptozotocin (STZ) rats model. After the treatment with avocado fruit extract, the elevated levels of blood glucose, glycosilated haemoglobin, blood urea and serum creatinine seen in the hyperglycaemic rats, reverted back to near normal. Determination of thiobarbituric acid reactive substances (TBARS), hydroperoxides and enzymatic and non-enzymatic antioxidants, confirmed the anti-oxidative potential of avocado fruit extract which, in turn, might be responsible for its hypoglycaemic potential. (Mahadeva and Adinew, 2011).

**CONCLUSION**

The present research showed that aqueous extract of P. americana Mill. leaf has PTP1B inhibitory activity that explain in part the mechanism underlying the antidiabetic effect. The more polar fraction (PaF10) showed the best inhibitory enzymatic activity. Future experiments are needed to clarify the chemical structures responsible of such biological activity.

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Inhibitory effect of Persea americana as therapeutic target for diabetes


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