A molecular connection of *Pterocarpus marsupium*, *Eugenia jambolana* and *Gymnema sylvestre* with dipeptidyl peptidase-4 in the treatment of diabetes

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Abstract

**Context:** *Pterocarpus marsupium* (PM) (Leguminosae), *Eugenia jambolana* (EJ) (Myrtaceae) and *Gymnema sylvestre* (GS) (Asclepiadaceae) are the most important medicinal plants in the Indian system of traditional medicine for the treatment of hyperglycemia.

**Objectives:** Dipeptidyl peptidase-4 (DPP-4) inhibitors are the emerging class of anti-diabetic agents. However, only few compounds are commercially available. Therefore, in the present study we tried to explore the naturally occurring PM, EJ and GS semi-standardized extracts for their potential DPP-4 inhibition in vitro and in vivo.

**Materials and methods:** DPP-4 inhibition was evaluated by in vitro inhibitory assay, and enzyme kinetics were calculated using one-phase exponential decay equation. Glucose load (2 g/kg) was administered to control and diabetic rats 30 min following extract administration (100, 200 and 400 mg/kg) orally once, and blood samples were withdrawn at 0, 0.5, 1, 1.5, 2 and 3 h to measure plasma active glucagon-like peptide-1 (GLP-1) levels.

**Results:** PM and EJ inhibit DPP-4 potently with IC₅₀ values of 273.73 ± 2.96 and 278.94 ± 6.73 μg/mL, respectively, compared to GS (773.22 ± 9.21 μg/mL). PM, EJ and GS exhibit long duration of action with enzyme inhibitory half-lives of 462.3, 317.2 and 153.8 min, respectively. Extracts significantly increase GLP-1 levels compared to negative control groups and peak GLP-1 level of action with enzyme inhibitory half-lives of 462.3, 317.2 and 153.8 min, respectively. Extracts significantly increase GLP-1 levels compared to negative control groups and peak GLP-1 level was observed at 2 h for PM and EJ, whereas for GS it was at 1.5 h.

**Discussion and conclusion:** Taken together, results suggest the extracts may have potent DPP-4 inhibitory action, and their hypoglycemic action attributed through an increase in plasma active GLP-1 levels.

Introduction

Diabetes is a chronic metabolic disorder present in almost all countries with an estimated number of 366 million in 2011 and may increase up to 552 million by 2030 (Nigel et al., 2011). Currently available agents for diabetes target either relative insulin deficiency or the insulin resistance, and have limitations such as increased risk of hypoglycemia, weight gain, gastrointestinal side effects and edema (Inzucchi, 2002). Discovery of incretin hormone, glucagon-like peptide-1 (GLP-1) lead a breakthrough in diabetes research by regulating postprandial glucose levels. The risk of hypoglycemia will be minimized due to insulinotropic actions of GLP-1 (Thomas et al., 2008). Other than insulin secretion, GLP-1 is also responsible for beta cell regeneration with reduced apoptosis (Drucker, 2003). Despite these facts, GLP-1 is inhibited by an enzyme called dipeptidyl peptidase-4 (DPP-4).

DPP-4 is a prolyl peptidase expressed in various types of cells including intestinal epithelial, liver, lung, placenta, kidney and renal proximal tubules. DPP-4 is involved in various functions including signal transduction, regulation of immunological and inflammatory response (Kikkawa et al., 2005). DPP-4 is known for transforming GLP-1 into an inactive metabolite, resulting in very short half-life of intact hormone (Drucker, 2003) and the GLP-1 effect will be renewed by inhibiting DPP-4 (Michel et al., 2008). Inhibition of DPP-4 leads to improved beta cell survival with increased neogenesis and reduced apoptosis (Rosenstock & Zinman, 2007). Researchers are active in the search for DPP-4 inhibitors that are available for the treatment of diabetes. Metformin is such a drug proved as a DPP-4 inhibitor (Lindsay et al., 2005).

The practice of herbal medicine in the field of metabolic disorders, especially diabetes, is growing. Herbs are a promising therapeutic approach in the treatment of diabetes as complementary and alternative medicine. In the present
study we explored naturally occurring *Pterocarpus marsupium* (PM) (Leguminosae), *Eugenia jambolana* (EJ) (Myrtaceae) and *Gymnema sylvestre* (GS) (Asclepiadaceae) semi-standardized extracts for their potential of DPP-4 inhibition *in vitro* and *in vivo*. The hypoglycemic action of selected plants has been proven well enough and the constituents responsible for hypoglycemic action are also recognized (Kanetkar et al., 2007; Manish et al., 2009; Manjeshwar et al., 2011). Though the selected plant extracts are available in various marketed ayurvedic formulations such as Bio-Gymnema, Diabecon and Diapal, and being used extensively, their molecular mechanism of hypoglycemic action is not explored clearly. So, present study was designed to explore the precise hypoglycemic mechanism of PM, EJ and GS.

**Materials and methods**

**Chemical, reagents and herbs used**

DPP-4 inhibitor screening assay kit was obtained from Cayman Chemicals (Ann Arbor, MI; Catalog no. 700210) and stored at −80°C. Active GLP-1 ELISA kit was purchased from Millipore (India; Catalog no. EGPLP-35K). PM heart wood extract containing 5% of pterostilbene, EJ fruit extract containing 20% of total polyphenols and 2% of ellagic acid, and GS leaves extract containing 25% total gymnemic acids was supplied by Natural Remedies Pvt Ltd (Bangalore, India). All other chemicals and reagents used in the study were of analytical grade and obtained from Sigma-Aldrich (Bangalore, India).

**Animals and treatment**

Male Wistar rats weighing 150–180 g were procured from the central animal house facility of JSS College of Pharmacy, Ooty, Tamil Nadu, India. Animals were housed in large spacious, hygienic polypropylene cages, well-ventilated and maintained at temperature 25 ± 3°C with relative humidity 44–56% along with light and dark cycles at 12 h. Animals were fed with rat-fed pellet supplied by Hindustan Unilever Ltd, Bangalore, India, and water *ad libitum*. All experiments were performed after obtaining prior approval from Committee for the Purpose of Control and Supervision on Experiments on Animals and Institutional Animal Ethical Committee (JSSCP/IAEC/PH.D/PH.COG/02/2011-2012).

Animals were allowed an acclimatization period up to a week and fasted overnight prior to the experiment. Eleven groups were divided consisting of six in each. Group I served as vehicle control, Groups II–XI were induced with diabetes by intraperitoneal injection of streptozotocin at a dose of 55 mg/kg body weight (Ravi et al., 2005) and assigned as follows: Group II served as negative control, Groups III–V treated with PM, Groups VI–VIII treated with EJ and Groups IX–XI treated with GS. Extracts were administered orally to their respective groups at a dose of 100, 200 or 400 mg/kg body weight.

**DPP-4 inhibition assay**

The assay was carried out using a DPP-4 inhibitor screening assay kit as per the manufacturer’s instructions. Briefly, the experiment was performed triplicate in a white half-volume 96-well plate. Thirty μL of assay buffer, 10 μL of DPP-4 enzyme, 10 μL of dimethoxysulphoxide (DMSO) and 50 μL of DPP substrate were added to 100% initial activity wells. Forty μL of assay buffer, 10 μL of DMSO and 50 μL of DPP substrate were added to background wells. Thirty μL of assay buffer, 10 μL of DPP-4 enzyme, 10 μL of extract (sample) and 50 μL of DPP substrate were added to inhibitor wells. The plate was incubated for 30 min at 37°C, and the fluorescence was recorded using a multimode microplate reader (Varioskan Flash, v.4.00.53) at excitation and emission wavelengths of 355 and 455 nm, respectively.

**Dissociation kinetics of DPP-4**

The dissociation kinetics was performed after 30 min pre-incubation of DPP-4 with 1000 μg/mL of each extract dissolved in assay buffer. The enzyme reaction was then initiated by adding DPP substrate and fluorescence intensities were measured every 5 min up to 10 h. Dissociation rate constants (*K_off*) and enzyme inhibitory half-lives (EI half-lives) were calculated from one-phase exponential decay equation fitted to the data points.

**Estimation of GLP-1 levels**

Glucose load (2 g/kg) was administered orally 30 min following extracts or vehicle administration, and blood samples were withdrawn at 0, 0.5, 1, 1.5, 2 and 3 h to measure plasma active GLP-1 levels. Plasma active GLP-1 level was estimated using commercially available ELISA kit according to manufacturer’s instructions. Briefly the assay was carried out in triplicate in a 96-well plate, 300 μL diluted wash buffer was added to each well, incubated for 5 min at room temperature and decanted. Two-hundred μL of assay buffer were added to blank wells and 100 μL of assay buffer were added to the remaining wells. One-hundred μL standards (GLP-1) were added in ascending order at concentrations of 2, 5, 10, 20, 50 and 100 pM. Next 100 μL quality control samples were added (QC1 and QC2) each and 100 μL samples were added in remaining wells. The plate was shaken gently for proper mixing and covered with plate sealer for an overnight incubation (24 h) at 4°C. After the incubation liquid was decanted, washed 5-times with 300 μL of wash buffer with 5 min incubation at each wash and finally the excess buffer was tapped out after the fifth wash. Two-hundred μL of detection conjugate was added to each well and incubated after 2 h incubation at room temperature. Then the plate was washed with 300 μL diluted wash buffer for 3-times and excess buffer was tapped out. Two-hundred μL of diluted substrate was added to each well and incubated for 20 min at room temperature in dark. Then 50 μL stop solution was added to each well in the same order as the substrate was added and incubated for 5 min at room temperature in the dark. Finally, fluorescence was recorded using multimode micro plate reader (Varioskan Flash, v.4.00.53) at excitation and emission wavelength of 355 and 460 nm, respectively.

**Statistical analysis**

The IC₅₀ values were calculated using regression analysis and expressed as mean ± SD. *K_0ff* and EI half-lives were calculated using Graph Pad Prism program (v.5.01) (La Jolla,
CA). ANOVA followed by the Bonferroni post-test was applied for GLP-1 levels and values were said to be significant when $p < 0.01$.

**Results and discussion**

GLP-1 is an endogenous peptide, synthesized and secreted from L-cells of the gastrointestinal tract (Perry & Greig, 2002). Unfortunately, GLP-1 has very short half-life (2 min) due to rapid degradation by DPP-4 (Yamazaki et al., 2006). Preclinical and clinical studies on DPP-4 inhibitors indicate their potential utility in the treatment of diabetes. Currently available DPP-4 inhibitors including sitagliptin, vildagliptin, saxagliptin, and several others are in the development phase.

In the course of development, we found DPP-4 inhibitors from herbal origin such as PM, EJ and GS. The present study demonstrates the molecular mechanism of PM, EJ and GS at their enzymatic level and characterization of their effect in vitro and in vivo.

Extracts showed concentration-dependent inhibition of DPP-4; potent inhibition was observed for PM and EJ with $IC_{50}$ values of 273.73 ± 2.96 and 278.94 ± 6.73 μg/mL, respectively, when compared to GS (773.22 ± 9.21 μg/mL) (Table 1). A clear time-dependent steady state approach was observed from the dissociation kinetic curves of the extracts (Figure 1). The initial reaction rates of DPP-4 with different concentrations of extracts were analyzed using one-phase exponential decay equation and data indicates that the selected extracts inhibit DPP-4 in competition to substrate binding sites. Inhibitors that bind tightly to the target are important for the pharmacological activity, as they inhibit enzyme function even if the circulatory free drug is cleared (Thomas et al., 2008). $K_{off}$ and EI half-lives of PM, EJ and GS were found to be 1.49, 2.18 and $4.50 \times 10^{-3}$/s and, 462.3, 317.2 and 153.8 min, respectively. Compared to GS, PM exhibited a 3-fold slower rate of decline with DPP-4 whereas EJ exhibited a 2-fold slower rate of decline. Irreversible binding was not observed with any of these extracts, yet PM and EJ were potent and longer acting DPP-4 inhibitors than GS.

The potency and duration of action of these extracts were further confirmed in an animal model of diabetes. A dose-dependent and significant ($p < 0.01$) increase in plasma active GLP-1 levels were observed following glucose load in extract-treated groups when compared to vehicle and negative control groups (Figure 2). The area under the curve (AUC) of PM and EJ shows peak GLP-1 levels at 2 h (Figure 3a and b), whereas GS at 1.5 h (Figure 3c). The insulin-secreting property of PM, EJ and GS (Ahmad et al., 1991; Ahmed et al., 2010; Sharma et al., 2008) was consistent with the elevated GLP-1 levels. Increase in plasma active GLP-1 levels might enhance insulin secretion due to DPP-4 inhibition by the extracts; indeed this pattern was not observed in vehicle and negative control groups. The elevated levels of GLP-1 following glucose load indicate that the secretion was glucose dependent and this potent long lasting in vivo action reflects in vitro EI half-lives.

Postprandial glucose level is regulated by DPP-4 inhibitors and free from side effects due to its glucose-dependent action (Ng & Kong, 2007). PM/EJ/GS administration before the

**Table 1. Effect of extracts on DPP-4 inhibition and kinetics.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>$IC_{50}$</th>
<th>$K_{off}$</th>
<th>EI half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. marsupium</em></td>
<td>273.73 ± 12</td>
<td>1.49</td>
<td>462.3</td>
</tr>
<tr>
<td><em>E. jambolana</em></td>
<td>278.94 ± 16</td>
<td>2.18</td>
<td>317.2</td>
</tr>
<tr>
<td><em>G. sylvestre</em></td>
<td>773.22 ± 19</td>
<td>4.50</td>
<td>153.8</td>
</tr>
</tbody>
</table>

$IC_{50}$ expressed in μg/mL. $K_{off}$ – dissociation constant and expressed as $\times 10^{-3}$/s. EI half-life – Enzyme inhibitory half-life and expressed in minute.

**Figure 1.** Dissociation kinetics of *P. marsupium* (PM), *E. jambolana* (EJ) and *G. sylvestre* (GS) with dipeptidyl peptidase-4 (DPP-4). DPP-4 was pre-incubated with each extract (1000 μg/mL), and the enzyme reaction was initiated by adding substrate. Fluorescence intensities were measured every 5 min for a total of 10 h. The values were the mean of triplicate studies.

**Figure 2.** Effect of extracts on plasma active glucagon-like peptide-1 levels in streptozotocin-induced diabetic rats. Graph represented as mean ± SD ($n = 6$). Two way ANOVA followed by Bonferroni post test was applied. ***$p < 0.001$, **$p < 0.01$ vs negative control. (a) *P. marsupium* (PM), (b) *E. jambolana* (EJ) and (c) *G. sylvestre* (GS).
meal may lead to an increase in GLP-1 levels by inhibiting DPP-4 which further controls post prandial glucose levels in diabetic patients. To our knowledge, for the first demonstration that PM, EJ and GS inhibit DPP-4 and increases GLP-1 levels, and lead to control glucose levels by secreting insulin from beta cells, the possible hypoglycemic mechanism.

Conclusion

In conclusion, with the aim of better understanding the biological role of herbal extracts, we showed that PM, EJ and GS inhibit DPP-4 and at the same time the data offers new perspectives for further development of herbal DPP-4 inhibitors. Finally, the hypoglycemic action of PM, EJ and GS might be attributed through DPP-4 inhibition. Further molecular docking studies on the constituents of PM, EJ and GS on DPP-4 (PDB id: 3C45) using the Schrodinger molecular modeling package is ongoing and will be reported in due course.

Declaration of interest

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References

Thomas L, Eckhardt M, Langkopf E, et al. (2008). (R)-8-(3-Amino-piperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinoxalin-2-ylmethyl)-3,7-dihydro-purine-2,6-dione (BI 1356), a novel xanthine-based dipeptidyl peptidase 4 inhibitor, has a superior potency and longer duration of action compared with other dipeptidyl peptidase-4 inhibitors. J Pharmacol Exp Ther 325:175–82.