Evaluation of some plants for potential dipeptidyl peptidase IV inhibitory effects \textit{in vitro}

Bazı bitkilerin \textit{in vitro} koşullarda; potansiyel dipeptidil peptidaz IV inhibitor etkilerinin değerlendirilmesi

**Abstract:** Objective: Dipeptidyl peptidase IV (DPP IV) is a serine amino (exo) peptidase which regulates various processes most notably plasma glucose homeostasis by cleaving incretin peptide hormones as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulin releasing polypeptide (GIP). Realization of the inhibition of this enzyme in controlling diabetes is one of the strategies adopted in recent years. The present study was designed to investigate the DPP IV inhibitory effects of sixteen plant having antidiabetic property in aqueous extracts in correlation with their protein content.

Methods: \textit{In vitro} DPP IV inhibition was evaluated by the specific inhibitory activity of plant aqueous extracts prepared without and with heat (60°C) treatment.

Results: Among the tested plants \textit{Vitis vinifera} L., \textit{Artemisia dracunculus} L., \textit{Prunus laurocerasus} L., \textit{Rubus caesius} L. and \textit{Olea europaea} L. extracts showed DPP IV inhibitory activity with respect to IC\textsubscript{50} values of 0.04-0.09 mg protein/ml. Kinetic analysis indicated that the inhibitor potency of \textit{A. dracunculus} extract was stronger than the other extracts.

Conclusion: The present study is the first report on screening and preliminary characterization of DPP IV inhibitory activity in aqueous extracts of selected antidiabetic medicinal food. This study could provide a new insight into DPP IV inhibitors from plants that could be useful for treatment of Type 2 diabetes.

**Keywords:** Type 2 diabetes, dipeptidyl peptidase IV, enzyme inhibition, serine protease, glycaemic control, medicinal plants

**Özet:** Amaç: Dipeptidil peptidaz IV(DPP IV); çeşitli proseslerin regüleasyonunda özellikle plazma glukoz dengesinin sağlanmasında önemli rol oynayan incretin peptid hormonlarının [glukagon benzeri peptid-1 (GLP-1) ve glukoz-bağımlı insulinotropik polipeptid (GIP)] yıkımından sorumlu bir serin amino (ekzo) peptidadır. Bu enzimin inhibisyonunun gerçekleştirilmesi ile diyabetin kontrol alıma almaması, son yıllarda benimsenen stratejilerin başında gelmektedir. Bu çalışmaların amacı, antidiyabetik özellikle sahip 16 bitkisinin DPP IV inhibisyon etkinliğinin farklı koşullarda hazırlanan sulu ekstraktlarında protein değerleri esas almakta belirlenmesidir.

**Metod:** DPP IV inhibisyonu, oda sıcaklığı ve 60°C’de hazırlanan bitki sulu ekstraktlarının spesifik inhibitor aktivitelerinin ölçülmesi ile değerlendirildi.

**Bulgular:** Test edilen bitkilerden \textit{V. vinifera} L., \textit{A. dracunculus} L., \textit{P. laurocerasus} L., \textit{R. caesius} L. ve \textit{O. europaea} L.’nın sulu ekstraktlarında DPP IV inhibitory aktivitesinin IC\textsubscript{50} değerleri 0.04-0.09 mg protein/ml olarak gözlemdi. Yapılan kinetik analizler ile \textit{A. dracunculus} ekstraktının inhibitör potansiyelinin diğer ekstralara göre daha güçlü olduğu belirlendi.

**Sonuç:** Bu çalışma ile seçilmiştir olan antidiyabetik tıbbi gidalardan sulu ekstraktlarında DPP IV inhibitory aktivitesinin taraması ve ön karakterizasyonu için ilk rapor oluşturulmuştur. Bu çalışma tip 2 diyet tedavisinde kullanılabilecek bitkiler için DPP IV inhibitörleri taramasma yönelik yeni bir bakış açısı sağlayacaktır.

*Corresponding author: Ali Zeytünlioğlu: Pamukkale University, Denizli Vocational School of Technical Sciences, Department of Electronic and Automation, Denizli, e-mail: azeytun@pau.edu.tr
Figen Zihnioğlu: Ege University, Faculty of Science, Department of Biochemistry, İzmir, e-mail: figen.zihnio glu@ege.edu.tr
Introduction

Type 2 diabetes (Non-insulin dependent diabetes mellitus: NIDDM) is a chronic metabolism disorder characterised by insulin resistance or the abnormal secretion of insulin [1]. Type 2 diabetes mellitus (T2DM) is one of major causes of morbidity all over the World [2]. Currently, 150 million people worldwide are considered to be diabetic and this number is expected to rise to 300 million in year 2025 [3]. The long term manifestation of this disease can result in the development of vascular disorders, such as retinopathy, neuropathy and angiopathy [4,5]. Numbers of alternative therapies are currently under development. One such approach is the inhibition of dipeptidyl peptidase IV, the major enzyme degrading the incretins in vivo [6]. Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) hydrolyses biologically active peptides that control critical functions, such as immune response, particular T cell activation, signal transduction and T cell proliferation and metabolic homeostasis [7,8]. The discovery of the role of enzymes in most important diseases such as Type 2 diabetes accelerated the design of potential pharmaceutical agents for the treatment of Type 2 diabetes mellitus. DPP IV rapidly inactivates the incretin hormones: glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) which serve as important prandial stimulator of insulin secretion and regulators of blood glucose control [9,10]. Inhibitors of the dipeptidyl peptidase IV reversibly block DPP IV mediated inactivation of these incretin hormones resulted in glycemic control. Because of this fact recently researchers focused on the development of DPP IV inhibitor compounds as a major new class of oral antidiabetic agents. Several classes of DPP IV inhibitors have been progressed in clinical development [11–13]. Some of them are dipeptide analogs of the natural substrates, mimicking their transition state. Inhibitors from natural sources have some advantages in comparison with those of synthetic origin such as low toxicity, high stability in physiological conditions and a wide variety of chemical structures for the design of new drugs [14]. In recent years many plants were screened for their antidiabetic potential [15]. The constituents that come under the category of polysaccharides, peptides, alkaloids, glycopeptides, triterpenoids, amino acids, steroids, xanthene, flavonoids, lipids, phenolics, cumarins, iridoids, alkyd disulphides, inorganic ions and guanidines were reported to have antidiabetic activity upon variety of mechanisms [16]. Among these, methanolic extracts of Mangifera indica L. leaves was tested in vitro for DPP IV inhibitory activity [17]. However there has not been a conducted research for screening of aqueous plant extracts capable of inhibiting dipeptidyl peptidase activity.

In the present study; we evaluated the screening and preliminary characterization of DPP IV inhibitory activity in aqueous extracts of selected traditional antidiabetic medicinal plants. Most of the plants tested in this study are part of dietary component, so there is less possibility of side effects caused by these plants.

Materials and Methods

All of the chemicals used were of molecular grades and obtained from commercial suppliers. Gly-Pro-p-Nitroaniline was purchased from Bachem (www.bachem.com).

Plant materials

Sixteen plants (Scientific, English, Turkish names and the parts used are listed in Table 1) were purchased from local markets in Izmir between July and September and then were identified by Dr. S. Gokhan Senol an expert on plant systematic at the Faculty of Science, Ege University Izmir.

DPP IV preparation from sheep kidney

Sheep kidneys from slaughterhouse of Tansas, Izmir, Turkey were homogenized using freeze-thaw under liquid nitrogen. Following extraction with 0.25% sucrose containing 50 mM Tris buffer, pH 8.0, the crude enzyme preparation was centrifuged at 4°C, 10000 g for 30 min. The supernatant was kept at -80°C until use. This study was approved by the Animal Research Ethical Committee of the Medical School, Ege University, Izmir, Turkey (2008 - 40).

Protein determination

The protein concentrations were determined according to...
the micro Bradford assay [18] using bovine serum albumin as standard.

**Preparation of plant extracts**

Aqueous extracts of selected plants known as antidiabetic (Momordica charantia, Teucrium chamaedrys, Cinnamomum verum, Prunus laurocerasus, Juglans regia, Artemisia dracunculus, Rubus caesius, Olea europaea) and serine protease inhibitor containing antidiabetic plants (Glycine max, Cucumis sativus, Daucus carota, Vitis vinifera, Hordeum vulgare, Triticum aestivum, Zea mays, Avena sativa) [19-34] were prepared as follows: Seed of plants used were coarsely powdered previously and then were homogenized in distilled water (0.5g/ml) at room temperature. Fresh fruit and leaves of plants used were homogenized in same conditions. Homogenates were centrifuged at 10000 g for 20 min and extracts were lyophilized (I). Second series (II) of plant extracts were also prepared by heating the extract for 30 min, at 60°C as was stated. The protein content of the lyophylizates (0.1 g/ml) were estimated and used for inhibition assays.

**DPP IV inhibition and kinetic studies**

Enzyme activity was monitored following an increase in absorbance at 405 nm resulting from the cleavage of the substrate Gly-Pro-pNA by DPP IV to release p-nitroaniline (pNA) in 96 well plates. Briefly, kidney DPP IV diluted in 0.1 M Tris buffer, pH 8.0 was incubated at 37°C for 15 min without and with appropriate amounts of plant extracts (0.025-0.01 mg protein/ml) in total volume of 100 µl. Finally 100 µl of substrate solution was added and absorbance was measured over 15 min at 405 nm in a microplate reader (Multiskan FC, Thermo Scientific). Controls without plant extracts were used as reference. For all tests the inhibition assay was performed in triplicate. One unit of specific inhibitory activity was defined as the amount of sample (in terms of protein concentration) needed to inhibit one unit of enzyme activity. Inhibitory ratio was calculated as follows:

\[
\text{Inhibition (\%)} = 1 - \left( \frac{\text{Activity}_{\text{sample}}}{\text{Activity}_{\text{control}}} \right) 
\]

A standard serine protease inhibitor (PMSF from Sigma, USA) was employed as positive control. Data are expressed as mean±SEM (n=3). IC₅₀ values were determined by linear regression of the dose-inhibition curves in linear range and defined as the amount of the plant extracts (in terms of protein concentration) needed to inhibit the 50% of control enzyme activity. The type of inhibition was examined among the most effective plants. DPP IV activity was measured with the increasing concentrations of Gly-Pro-pNA (0.05-0.75 mM) in the absence and presence of most effective plant extracts. The type of inhibition and kinetic constants were calculated on the basis Lineweaver-Burk plots using Sigma Plot 10.0 software.

**Table 1:** List of the studied plants commonly consumed by Turkish people.

| Scientific name                      | Family name     | English name       | Local name          | Part used |
|--------------------------------------|-----------------|--------------------|---------------------|-----------|
| Glycine max L. Merr.                 | Leguminosae     | Soybean            | Soya fasulyesi      | Seed     |
| Cucumis sativus L.                   | Cucurbitaceae   | Cucumber           | Salatalik           | Fruit    |
| Daucus carota L.                     | Umbelliferae    | Wild carrot        | Havuc               | Tuber    |
| Vitis vinifera L.                    | Vitaceae        | Grape              | Uzum                | Fruit    |
| Hordeum vulgare L.                   | Gramineae       | barley             | Arpa                | Seed     |
| Triticum aestivum L. Thell           | Gramineae       | Wheat              | Bugday              | Seed     |
| Zea mays L.                          | Gramineae       | Sweet corn         | Msir                | Seed     |
| Avena sativa L.                      | Gramineae       | Oat                | Yulaf               | Seed     |
| Momordica charantia L.               | Cucurbitaceae   | Bitter melon       | Kudret narı         | Fruit    |
| Teucrium chamaedrys L.               | Labiatae        | Wild germander     | Bodur mahmut        | Leaf     |
| Cinnamomum verum J. Presl.           | Lauraceae       | Cinnamon           | Tarçın              | Bark (sticks) |
| Prunus laurocerasus L.               | Rosaceae        | Cherry laurel      | Laz kirazi          | Fruit    |
| Juglans regia L.                     | Juglandaceae    | Walnut             | Ceviz               | Leaf     |
| Artemisia dracunculus L.             | Asteraceae      | Estragon           | Tarhun              | Leaf     |
| Rubus caesius L.                     | Rosaceae        | Dewberry           | Bogürtlen           | Leaf     |
| Olea europaea L.                     | Oleaceae        | Olive              | Zeytin              | Leaf     |
Results

In order to detect new sources of natural inhibitors of DPP IV from plant origin, we searched inhibitory activity in aqueous extracts of 16 plants which are categorized as antidiabetic and serine protease inhibitor containing antidiabetic. It should be noted that while generally cold and hot water extracts are most commonly used in the traditional method of preparing medicines in Ayurveda. Table 2 shows the results of the screening aqueous extracts of 16 medicinal plant species treated with and without heat. Depending on the preparation of crude extracts, all plant samples seemed to have potent specific inhibitory activities in terms of protein concentration except *M. charantia* L.

Preliminary characterization of the inhibitory activity involved to study the effect of different doses on the inhibitory activity to corroborate the presence of a molecular entity characterized by a dose dependent effect. IC$_{50}$ values were calculated as a measure of inhibitory effectiveness for plants showing the most specific inhibitory effect (Table 2).

Thermal treatment was more effective for serine protease inhibitor containing plants especially for *D. carota* and *V. vinifera*. In the case of antidiabetic plants, thermal treatment had significant effect only for *A. dracunculus*. The mixed results suggested that the inhibitory molecule is resistant to heat treatment and induce the activation of target inhibitor compounds. On the other hand *P. laurocerasus, R. caesius* and *O. europea* extracts that are prepared without heat treatment had the highest specific inhibitory activity with low thermal stability. Among all obtained data indicated clearly *D. carota, V. vinifera, A. dracunculus, P. laurocerasus, R. caesius* and *O. europea* extracts could be promising for an effective DPP IV inhibitor due to their IC$_{50}$ values besides exhibiting dose-dependent inhibitory activities. *O. europaea* turned out to be the strongest inhibitor against DPP IV compared to positive control (PMSF). *D. carota, V. vinifera, A. dracunculus, P. laurocerasus, R. caesius* and *O. europea* were further investigated to explore the kinetic characteristics. Kinetic analysis using Lineweaver-Burk plots revealed that all of the plants that displayed high inhibitory activity did so through a partial mixed type mode of inhibition (α<β) comprising both partial competitive and partial noncompetitive components (Figure 1) that substrate bound EIS complex maintains a reduced level of catalytic activity.

Kinetic analysis indicated that Ki values for *A. dracun-

### Table 2: Dipeptidyl Peptidase IV Inhibitory Activity of Some Antidiabetic Medicinal Plants.

| Species                        | Protein (mg/ml) | Specific Inhibitory Activity (U/mg) | IC$_{50}$ (mg/ml) |
|-------------------------------|----------------|------------------------------------|------------------|
|                               | I             | II                                | I                | II              | I                | II              |
| Serine protease inhibitor containing anti-diabetic plants |                |                                    |                  |                 |                  |                 |
| *Glycine max*                 | 7.9±0.03      | 2.9±0.6                           | NI               | 2.1±0.5         | ND               | 0.3±0.02        |
| *Cucumis sativus*             | 0.7±0.07      | 0.4±0.04                          | 5.9±0.6          | 3.2±0.4         | 0.1±0.08         | ND               |
| *Daucus carota*               | 1.2±0.09      | 0.5±0.01                          | 0.8±0.1          | 9.6±0.8         | ND               | 0.07±0.04       |
| *Vitis vinifera*              | 0.1±0.07      | 0.08±0.02                         | NI               | 15±1.4          | ND               | 0.05±0.01       |
| *Hordeum vulgare*             | 4.0±0.43      | 1.2±0.3                           | 0.6±0.05         | 2.4±0.9         | ND               | 0.2±0.03        |
| *Triticum aestivum*           | 13±0.83       | 3.2±0.2                           | 1.9±0.5          | 1.8±0.4         | ND               | 0.4±0.02        |
| *Zea mays*                    | 8.6±0.60      | 1.8±0.9                           | NI               | 1.8±0.4         | ND               | 0.5±0.03        |
| *Avena sativa*                | 2.9±0.2       | 1.3±0.6                           | NI               | 1.8±0.4         | ND               | 0.4±0.02        |

| Anti-diabetic plants          |                |                                    |                  |                 |                  |                 |
| *Momordica charantia*         | 0.6±0.06       | 0.2±0.04                          | NI               | NI              | ND               | ND              |
| *Teucrium chamaedrys*         | 0.7±0.04       | 0.6±0.04                          | 3.2±0.8          | 4.1±0.7         | 0.2±0.04         | 0.17±0.02       |
| *Cinnamomum verum*            | 5.4±0.14       | 1.7±0.07                          | 3.1±0.2          | 1.4±0.4         | 0.3±0.04         | ND              |
| *Prunus laurocerasus*         | 0.2±0.05       | 0.05±0.02                         | 9.8±1.9          | 3.9±0.9         | 0.07±0.02        | 0.15±0.04       |
| *Juglans regia*               | 1.6±0.09       | 0.8±0.04                          | 3.7±0.2          | 4.3±0.4         | 0.2±0.03         | 0.17±0.08       |
| *Artemisia dracunculus*       | 5.4±0.19       | 0.5±0.07                          | 3.6±0.5          | 8.3±1.3         | 0.2±0.03         | 0.09±0.06       |
| *Rubus caesius*               | 5.3±0.12       | 0.8±0.05                          | 11.5±1.1         | 2.3±0.7         | 0.0±0.01         | ND              |
| *Olea europaea*               | 0.7±0.05       | 0.2±0.03                          | 15.3±1.6         | 4.4±0.9         | 0.0±0.01         | ND              |
| PMSF(control)                 | 12.8±2.0       | 0.06±0.01                         |                  |                 |                  |                 |

Data are means of triplicates; NI: No enzyme inhibition; ND: Not determined; 'Inhibitory activity was defined as the amount of protein needed to inhibit one unit of enzyme activity; †0.1 g plant extract lyophilizate/ml; I and II without and with heat treatment (60°C), respectively.
culus and D. carota were 0.34 and 89.2 µg/ml, respectively, showing that the potency of inhibitor compounds in A. dracunculus extract was approximately 260 times stronger than D. carota. Other extracts possessing similar results with a 3-5 fold weakest than the A. dracunculus (Table 3).

**Discussion**

Peptidases are important for many biochemical and physiological processes directly and indirectly such as digestion, fertilization, differentiation, cell signaling, wound
healing, immunological defense, apoptosis and related diseases. Specific inhibitors of such peptidases are emerging with promising therapeutic uses in the treatment of diseases such as cancers, inflammatory diseases and metabolic disorders. A serine protease dipeptidyl peptidase IV has become a validated target for the treatment of Type 2 diabetes, with several inhibitors are classified as peptide derived and non-peptide inhibitors [35]. In general, various peptidase inhibitors have been searched from plants, microorganisms and animal sources by means of their pharmacological use. Plants are an abundant source of biologically active compounds acting as inhibitors for various enzymes. The aim of the present study was to investigate the DPP IV inhibitory activity from medicinal plants known for their anti-diabetic properties. To date no reports for DPP IV inhibition from the aqueous extracts of these plants exists in the literature. On the other hand, the methanol extract of M. indica L. has been shown to inhibit DPP IV activity exhibiting competitive type of inhibition [17]. However, besides the extraction medium, the present study was conducted to target peptide/protein contents of the extracts. Specific inhibitory activities, IC50 values and kinetic parameters were calculated on the basis of their protein concentrations that makes this study incomparable due to the lack of structural data. On the other hand various inhibition types such as partial and mixed type and competitive was reported for inhibitor compounds and peptides [13,36]. The Ki values and inhibitory potency we obtained allows discrimination between crude anti-diabetic plant extract inhibitors for DPP IV and clarify the correlation between compound potency and stability of anti-diabetic medicinal plants as well as mechanism of action of well-known anti-diabetic plants by means of their DPP IV inhibitory activity. Herbs act against diabetesthrough various mechanisms such as insulin secretion, reducing insulin resistance, insulinomimetic effect, delaying glucose absorption, inhibitors of intermediary metabolism etc. [37]. DPP IV inhibitors are a new class of oral lowering agents for the treatment of type 2 diabetes. Although the absolute specificity of DPP IV inhibitors was not clear, several DPP IV inhibitors have shown to increase insulin secretion and reduce blood glucose level after glucose challenge [12,38–40]. Thus, DPP IV inhibition represents an attractive strategy to develop antidiabetic agents.

Our findings indicate that V. vinifera, A. dracunculus, P. laurocerasus, R. caesius and O. europaea extracts showed specific potent DPP IV inhibitory activity in vitro. Further studies are warranted to isolate and characterize the inhibitor compounds. Then their potential for therapeutic use in prevention and treatment of hyperglycemia and diabetes should be confirmed by in vivo studies.

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Conflict of Interest: The authors have no conflict of interest.

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