



Biolog. Journal of Armenia, 1 (70), 2018

IN VITRO AND IN VIVO INFLUENCE OF PLANT PREPARATIONS ON DIPEPTIDYL PEPTIDASE IV AND ADENOSINE DEAMINASE ACTIVITIES

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Dipeptidyl peptidase IV (DPPIV) and adenosine deaminase (ADA) are multifunctional enzymes, involved in different physiological processes. Often a problem of their regulation appears. Particularly, the inhibition of both enzymes considered beneficial at type 2 diabetes. This work describes the influence of ethanol extracts and constituents from several plants on DPPIV and ADA activities. The IC_{50} values of extracts from pellicles of walnut kernel, rose petals, melilot, leaves of pistacia, grape, sorrel and blackberry in the *in vitro* inhibition of ADA from bovine lung were in the range of 0.23-1.25 mg/ml. In inhibition of DPPIV from bovine kidney, the IC_{50} values of the extracts from blackberry leaves, pellicles of walnut kernel and rose petals were between 0.13 and 0.63 mg/ml. The IC_{50} values of phenol glycoside fractions from rose petals and grape leaves in DPPIV inhibition (0.029 mg/ml) were by one order smaller than for ADA (0.34 mg/ml). In the *in vivo* experiments, the increasing of ADA and DPPIV activities in the blood plasma of the streptozotocin injected rats (40 mg/kg of body weight) was proved. In these animals the extract from walnut kernel pellicles effectively inhibited the activities of both enzymes.

Adenosine deaminase–dipeptidyl peptidase IV– enzymatic activity regulation– plant extracts

Դիպեպտիդիլպեպտիդազ IV-ը (ԴՊՊ IV) և ադենոզինդեամինազը (ԱԴԱ) բազմաֆունկցիոնալ ֆերմենտներ են, ներգրավված տարբեր ֆիզիոլոգիական պրոցեսներում: Բազմաթիվ պաթոլոգիաներում գրանցվել է դրանց ակտիվության աճ: Ուստի, հաճախ ֆերմենտների կարգավորման խնդիր է առաջանում, մասնավորապես, 2-րդ տիպի շաքարախտի ժամանակ: Այս աշխատանքում նկարագրվում է որոշ բույսերի էթանոլային թուրմերի և բաղադրիչների ազդեցությունը նշված ֆերմենտների վրա: Ցուլի թոբի ԱԴԱ-ի ակտիվության *in vitro* ընկճման ժամանակ իշառվույտի, ընկույզի միջուկի թաղանթի, վարդի ծաղկաթերթիկների, պիստակի, խաղողի, ավելուկի և մոշի տերևների թուրմերի IC_{50} արժեքները գտնվում էին 0.23-1.25 մգ/մլ տիրույթում: Մոշի տերևների, ընկույզի միջուկի թաղանթի, վարդի ծաղկաթերթիկների թուրմերով ցուլի երիկամների ԴՊՊIV-ի արգելակման IC_{50} արժեքները 0.13-0.63 մգ/մլ միջակայքում էին: Վարդի ծաղկաթերթիկների և խաղողի տերևների ֆենոլագլիկոզիդային ֆրակցիաների IC_{50} արժեքները ԴՊՊIV-ի դեպքում (0.029 մգ/մլ) մեկ կարգով ավելի փոքր էին, քան ԱԴԱ-ի դեպքում (0.34 մգ/մլ): Հաստատվել է ստրեպտոզոտոցին ներարկված (40 մգ/կգ կենդանու կշիռ) առնետների արյան պլազմայում ԴՊՊIV-ի և ԱԴԱ-ի ակտիվության աճը: Այս կենդանիների մոտ ամենաարդյունավետ *in vivo* արգելակիչ հանդիսացավ ընկույզի միջուկի թաղանթի թուրմը:

Ադենոզինդեամինազ – դիպեպտիդիլպեպտիդազ IV – ֆերմենտային ակտիվության կարգավորում – բուսական թուրմեր

Дипептидилпептидаза IV (ДППИВ) и аденозиндеаминаза (АДА) –многофункциональные ферменты, вовлеченные в различные физиологические процессы. При ряде патологий наблюдается значительное увеличение их активности. Вследствие этого часто возникает необходимость ингибирования активности этих ферментов. В частности, желательна ингибирование ДППИВ и АДА при сахарном диабете второго типа. В данной работе представлено воздействие этанольных экстрактов и компонентов некоторых растений на активности ДППИВ и АДА. Значения IC_{50} при *invitro* ингибировании активности АДА из легких быка экстрактами донника лекарственного, пленки ядра грецкого ореха, лепестков розы, листьев фиштакшоваго дерева, винограда, конского щавеля и ежевики находились в пределах 0.23-1.25 мг/мл. IC_{50} экстрактов пленки ореха, листьев ежевики и лепестков розы при *invitro* ингибировании ДППИВ из почек быка находились в пределах 0.13-0.63 мг/мл. Значения IC_{50} для фенолгликозидов из лепестков розы и листьев винограда в случае ДППИВ (0.029 мг/мл) были на порядок меньше, чем для АДА (0.34 мг/мл). В опытах *invivo* подтверждено возрастание активности ДППИВ и АДА в плазме крови экспериментальных крыс после инъекции стрептозотоцина (40 мг/кг веса). У этих животных активность обоих ферментов наиболее эффективно подавлял экстракт пленки орехового ядра.

Аденозиндеаминаза – дипептидилпептидаза IV – регулирование ферментативной активности – растительные экстракты

Adenosine deaminase (ADA, E.C. 3.5.4.4) catalyzes deamination of (deoxy)adenosine (Ado) and plays a critical role in maturation and differentiation of lymphoid cells, maintaining the effective immune, neurological and vascular systems [4]. Two molecular forms of ADA are as follows: a small, intracellular SADA, and a large LADA, formed as a complex of SADA and DPPIV/CD-26 [6]. The located on the cell membranes and circulating LADA catalyzes deamination of Ado in the intercellular medium. Adenosine receptors (ARs) were also identified as ADA anchoring proteins leading to activation of T-cells [5]. The costimulatory effects promoted by the ADA-DPPIV/CD26 complex consist in the inducing of IL-6 production, INF- γ and TNF- α secretion, enhancing the pro-inflammatory response [16].

A multifunctional dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) is a ubiquitous and unique serine protease removing N-terminal dipeptides from the polypeptides and proteins, containing proline or alanine on the penultimate position. This enzyme truncates many cytokines, chemokines and peptide hormones. It is identical to CD26, an antigen of activated human T lymphocytes, involved in regulatory and co-stimulatory events in the immune system [3, 10]. The clinical studies of the role of DPPIV and of the specific inhibitors paved the way to novel therapeutic concepts in many pathologies such as diabetes [22]. The membrane-bound form of DPPIV is expressed in many tissues on T-, B-, natural killer, hematopoietic progenitor and stem cells and macrophages. The soluble form of DPPIV is circulating in blood plasma and is detected in urine, bile, semen, cerebrospinal, pleural and synovial fluids [3, 10].

The described importance of concerted and individual functions of DPPIV and ADA in humans, their association with different vital systems forced to study of the ways of their regulation. Particularly, this problem concerns the diabetes mellitus.

The glucagon-like peptide-1 (GLP-1) (7-36) is insulinotropic incretin hormone and a most potent therapeutic agent for treatment of type 2 diabetes (T2D). However, DPPIV converted it to inactive GLP-1(9-36) [7] and the inhibition of DPPIV is one of the strategies to prolong its antidiabetic activity [9]. At present, the inhibitors of DPPIV are under development in preclinical and clinical studies, and several of them are marketed as anti-diabetic drugs [17]. As it is known, the serum level of ADA in T2D patients was increased reliably [12]. It was elevated in the streptozotocin-injected rats.

The increase of ADA can result in decreasing of the Ado level and in development of insulin resistance [18].

The detection of natural inhibitors of DPPIV and ADA could be beneficial for treatment of T2D. There is preclinical evidence for the efficacy of plants as hypoglycemic agents or in the management of diabetic complications [8]. Medicinal herbs contain diverse bioactive compounds having positive effects on insulin production. Metformin, a first line drug for T2D, is based on a biguanide compound from the antidiabetic French lilac [2].

Earlier, we demonstrated the inhibition of DPPIV and ADA activities by aqueous extracts from Armenian Highland plants [13]. The transmission and scanning electron microscopy study [11] proved the ability of the ethanol extract of rose petals and of its phenole glycoside fraction to hinder the fibrillation of peptide hormone of pancreas, amylin (one of the causes of T2D development). The decreasing of amylin aggregation and the *in vitro* protection of islet β -cells from death in the presence of aggregated amylin by the ethanol extracts and fractions from several plants were demonstrated [20, 21]. These results prove the probability of usefulness of herbal constituents in prevention and treatment of T2D.

This work describes the *in vitro* and *in vivo* influence on DPPIV and ADA activities of ethanol extracts from several plants and their constituents. For study, we used the plants which traditionally are used in folk medicine and/or as food in Armenian cousin, but are not studied by researchers before.

Materials and methods. The leaves of grape (*Vitisvinifera*), sorrel (*RumexConfertus*), pistacia (*Pistacia atlanticaDesf.*) and blackberry (*RubusCaesius*), rose petals (*Rosa damascena*), aerial parts of melilot (*Melilotusofficinalis*) and walnut (*Juglansregia*) were collected from Armenian Highland and dried in the shade. A voucher specimen has been deposited in the herbarium of the Botanical Department of Yerevan State University (Dr. NarineZaqaryan). The ethanol extract preparation, identification of constituents, fractionation by gel-filtration and characterization of fractions by chemical analyses, thin layer chromatography and optical absorbance in UV-Vis region are described earlier [1].

For *in vitro* experiments, ADA and DPPIV were purified from bovine lung and kidney, respectively [14, 19]. In the assay of their activities, Ado and Gly-Pro p-nitroanilide toluenesulfonate salt, purchased from Sigma Ltd. USA, were used as substrates.

In the *in vivo* experiment, 30 laboratory rats were divided to six groups. One of them was kept as untreated control. In 25 animals T2D model was induced by injection of streptozotocin (STZ, AppliChem GmbH, Germany) in dose of 40 mg/kg of body weight. One group was kept as STZ-control. The animals of four groups were fed orally three times per a week with the ethanol extracts from walnut kernel pellicles (WP), rose petals (RP), leaves of grape (GL) and sorrel (SL), indose of 400 mg/kg of body weight. This dose was chosen as most suitable after preliminary testing of doses between 150-400 mg/kg. In four weeks, per 1 ml of blood was taken cardiopunctually using heparinized syringe. DPPIV and ADA activities were assayed in the blood plasma.

Data analyses were performed using the statistical software InStat, version 3 for Windows (GraphPad Software, Inc., SanDiego, CA, USA). Specific differences were tested using Student's two-tailed *t*-test. The data showing $P < 0.05$ were considered as statistically authentic. Results were expressed as mean \pm s.e.m.

Results and Discussion. The *in vitro* effects of plant preparations on the activities of DPPIV and ADA, purified from bovine kidney and lung, respectively, were studied. From the concentration dependences, the IC_{50} values of the ethanol extracts and isolated fractions from plants in inhibition of DPPIV and ADA were estimated (tab. 1). These data evidence that IC_{50} values of the extracts from WP, pistacia and blackberry leaves in inhibition of two enzymes are in mutual conformity. IC_{50} in inhibition of DPPIV by

extract from RP is by one order smaller than in the case of ADA. The extracts from melilot, SL and GL influenced on the activities of two enzymes differently. Their IC₅₀ values in the case of ADA are in line with the other extracts, but these three extracts did not inhibit DPPIV. Moreover, the extract from melilot somewhat activated DPPIV.

Table 1. IC₅₀ values of plant preparations in inhibition of DPPIV and ADA activities

IC ₅₀ ,mg/ml ± s.e.m.			
ethanol extract from:		ADA	DPPIV
1	walnut kernel pellicles	0.23 ± 0.005	0.20 ± 0.01
2	pistacia leaves	0.29±0.014	0.39 ± 0.03
3	grape leaves	0.36 ± 0.03	>100
4	melilot	0.57± 0.014	Light activation
5	sorrel leaves	0.68 ± 0.06	>100
6	blackberry leaves	0.94± 0.09	0.63 ± 0.14
7	rose petals	1.25 ± 0.09	0.13 ± 0.01
Phenolic fractions from:			
8	rose petals	0.33 ± 0.02	0.029 ± 0.005
9	grape leaves	0.35 ± 0.05	0.029 ± 0.003

The IC₅₀ values of PhG from RP and GL in inhibition of the enzymes are very close one to other. In the case of DPPIV they were by one order smaller of that for ADA. It should be noted that in the identical experiments, the PhGs and the emodine from SL did not affect the activity of DPPIV.

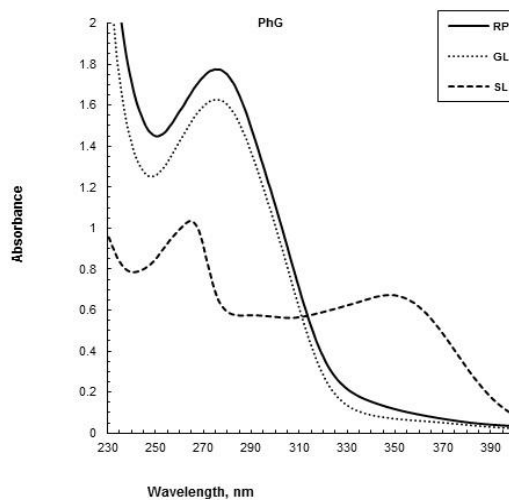


Fig. 1. The spectra of PhG fractions from RP, GL and SL.

Fig. 1 shows the optical absorption spectra of PhGs from three plants. It is interesting, that the spectrum of PhGs from SL, which did not affect the activity of DPPIV strongly differs from the spectra of PhGs from RP and GL, which inhibited DPPIV with close effectivity (IC₅₀ = 0.029 mg/ml). Obviously, the constituents of the last two chemically differ from those of PhG from SL. Comparison with the literature data [15] evidence that the spectra of GL and RP PhGs are similar to the spectra of the tannins' class flavon-3-ole ((+) – catechin)s. The absorbance of SL PhG is characteristic for monomeric flavonols and oligomeric procyanidins, the absorption band at 320-350 nm evidences the involvement of phenol oxides, flavonones and flavananes.

The dependences of DPPIV activity on the concentration of GL PhG were investigated at two concentrations of the substrate. The graphical analyses manifested the competitive nature of inhibition with K_i equal to 1.35 $\mu\text{g/ml}$.

The *in vivo* effects of the water suspensions of dried ethanol extracts from WP (150 mg/kg of body weight), GL (300 mg/kg) and SL (400 mg/kg) on the activities of DPPIV and ADA in the plasma of healthy rats were studied. Three groups of normal laboratory rats, per three animals in each, were fed with these extracts for 4 weeks (twice in a week). The doses were chosen taking into account their IC_{50} values in the *in vitro* inhibition of ADA (0.23 mg/ml < 0.36 mg/ml < 0.68 mg/ml, tab. 1).

The fourth group served as a control. In the end of the experiment, the activities of ADA and DPPIV were determined in the blood plasma of animals. In fig. 2, A and B the results, expressed in percentage of the control animals, are presented. The activity of ADA in the groups, fed with plant extracts, was lower than in the control group, but this decreasing was not in accordance with their *in vitro* IC_{50} values. The activity of DPPIV in the groups, fed with plant extracts, was higher than in the control. In spite of low IC_{50} value of the extract from WP (0.20 mg/ml, tab. 1), DPPIV activity in the group, which received this extract, was the highest.

Hence, the *in vivo* influences of three ethanol extracts on the ADA and DPPIV activities in blood plasma of healthy rats were strongly different and were not in accordance with the *in vitro* inhibition of the enzymes.

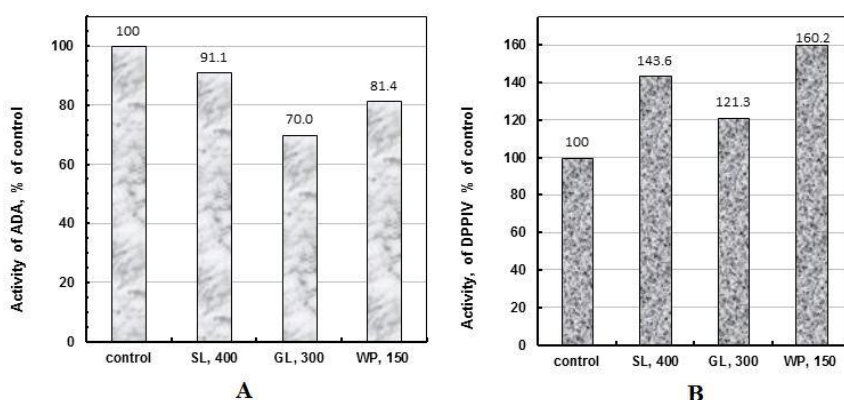


Fig.2. Activities of ADA (A) and DPPIV (B) in the blood plasma of healthy rats, fed for 4 weeks with the water suspensions of the ethanol extracts from WP, GL and SL.

In the next experiments, the *in vivo* effects of STZ and herbal extracts on the activities of DPPIV and ADA were studied. Four groups of STZ-injected animals were fed orally in the dose of 400 mg/kg of body weight for a month by the ethanol extracts from:

a) RP, which *in vitro* effectively inhibited DPPIV ($IC_{50} = 0.13 \pm 0.01$ mg/ml), but slightly inhibited ADA ($IC_{50} = 1.25 \pm 0.09$ mg/ml);

b) GL, which *in vitro* inhibited ADA ($IC_{50} = 0.36 \pm 0.03$ mg/ml), but did not influence on the DPPIV activity ($IC_{50} > 100$);

c) WP, which *in vitro* inhibited both ADA ($IC_{50} = 0.23 \pm 0.005$ mg/ml) and DPPIV ($IC_{50} = 0.20 \pm 0.01$ mg/ml);

d) SL which *in vitro* moderately inhibited ADA ($IC_{50} = 0.68 \pm 0.06$ mg/ml), but did not influence on the activity of DPPIV ($IC_{50} > 100$ mg/ml).

At the end of the experiment, DPPIV and ADA activities were determined in the blood plasma. Table 2 shows the ADA activities in 6 animal groups: untreated, STZ-control and 4 groups, which received the plant extracts. The data are presented in the percentage of the activity in the group of untreated animals. In accordance with the literature data [12], the activity of ADA in the blood of animals in the STZ-control group increased relative to the untreated group by 42 % (P<0.05). In the STZ-injected animals, which received the ethanol extracts from WP, RP, SL and GL, the ADA activity was inhibited *in vivo* moderately, down to 60-80 % of the untreated group (P>0.05). These values are significantly lower of that in STZ-control group and consist 45-55 % of it (P<0.05).

Table 2.The activity of ADA in 6 animal groups

Group	untreated	STZ-cont	RP, 400	GL, 400	WP, 400	SL, 400
% of control±s.e.m.	100±9.1	142±12.03	73±7.6	78±8.3	64±12.3	73±8.0
P vs untreated		*P<0.05	P>0.05	P>0.05	P>0.05	P>0.05
P vs STZ-cont			*P<0.05	*P<0.05	*P<0.05	*P<0.05

Tab.3 shows the activitis of DPPIV in the same groups. The activity of DPPIV in the STZ-control group is higher of that in the untreated animals by 24 % (P<0.05). In the animals, fed by the extracts from GL and SL, which did no inhibit DPPIV *in vitro* (tab. 1), the DPPIV activities were close to that in the STZ-control group. Surprisingly, in the group, fed by the extract from RP with the best *in vitro* inhibition (IC₅₀ = 0.13 mg/ml), the *in vivo* activity was higher than in STZ-control group.

Table 3. The activity of DPPIV in 6 animal groups

Group	untreated	STZ-cont	RP, 400	GL, 400	WP, 400	SL, 400
% of control ±s.e.m.	100±4.3	124±3.9	138±3.8	106±3.7	70±10.1	123±6.3
P vs untreated		*P<0.05	**P<0.01	P>0.05	P>0.05	P>0.05
P vs STZ-cont			P>0.05	P>0.05	**P<0.01	P>0.05

Only in the blood of animals, fed with the extract from the WP (*in vitro* IC₅₀ = 0.20 mg/ml), DPPIV activity was inhibited statistically significantly, down to 56 % of the STZ-control group (P<0.01).

The IC₅₀ values of several plant extracts (and two PhG fractions) evidenced moderate and significant values in inhibition of ADA *in vitro*. Their capabilities to inhibit DPPIV *in vitro* were different.

In the healthy animals, the ADA activity under action of some of the studied ethanol extracts *in vivo* was moderately lower of that in the control animals, but the activity of DPPIV was surprisingly higher than in the control animals.

Our study proved the increasing of ADA and DPPIV activities in the blood plasma of the STZ-injected animals. In the STZ-injected animals fed by plant extracts, the ADA activity was inhibited *in vivo* significantly, down to 45-55 % of the STZ-injected animals (P<0.05).

The extract from WP demonstrated one of the lowest IC₅₀ values in inhibition of DPPIV *in vitro* (0.20 ± 0.01 mg/ml). In the *in vivo* study, it inhibited the activity of

DPPIV down to 56 % of the activity in the STZ-injected group ($P < 0.01$). This is the best effect observed in our study.

It is worthy to note, that the extract from WP demonstrated the lowest IC_{50} values for both enzymes (0.23 and 0.20 mg/ml for ADA and DPPIV respectively). In the *in vivo* experiment, this extract also demonstrated the best inhibition of ADA and DPPIV (down to 60 and 70 % of untreated or 45 and 56 % of STZ-control groups, respectively). We can conclude, that the active constituent(s) in this extract (contrary to those from RP) is (are) stable against the digestion processes in the animals and inhibited the enzymes *in vivo* rather effectively, in accordance with the *in vitro* inhibition. Hopefully, the obtained results will allow considering usefulness of some Armenian Highland plants in the treatment of diabetes mellitus as co-drugs.

Acknowledgment

This work was supported by State Committee Science MES RA, in the frame of the research project № SCS 15T-1F164.

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Received on 29.09.2017