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HERBAL INFUSIONS AS INHIBITORS OF TRYPSIN ACTIVITY IN WATER AND POLAR ORGANIC SOLVENT–WATER SYSTEMS

A. Kh. JAMAL [1*], A. Q. MOHAMMED [1**], A. H. RASHEED [1***], A. R. AL-RUBBAWI [1****], G. A. SHAHINYAN [1,2*****]

[1] *Eurasia International University, Armenia*
[2] *Research Center of Chemistry, Laboratory of Ecological Safety, YSU, Armenia*

The inhibition of digestive enzyme trypsin by polyphenolic compounds derived from mountainous herbal infusions, as well as the effect of polarity and acidity of the media on this process were studied by virtue of UV-Vis absorption spectroscopy at 293.15 *K*. Mountainous herbal infusions (thyme, peppermint) are well known due to their biomedical significance and wide use as a beverage. The binding constant (K_b) and standard Gibbs energy change (ΔG^0) were calculated. The obtained results show that polyphenols extracted from herbal infusions significantly inhibit trypsin. Moreover, the effect of thyme infusion is stronger compared with that of peppermint. The results show that the change of both polarity and H-bonding ability of polar organic cosolvent significantly alters the value of K_b, indicating that H-bonds and electrostatic interactions are the main driving forces in these interactions.

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***Keywords*:** trypsin, polyphenolic compounds, thyme infusion, peppermint infusion, polar organic cosolvent, acidic media.

Introduction. Protease inhibitors are one of the most promising and investigated subjects for their role in pharmacological studies, biological functions, and medical benefits [1]. The binding of small molecular drugs to proteins is in the focus of many researchers. These studies may reveal the potential application of small molecules as drugs [1–3]. Trypsin is a typical serine protease, which is excreted by the pancreas into the small intestine. Trypsin plays an important role in physiological processes such as apoptosis, hemostasis, signal transduction, reproduction, and immune response [4]. Therefore, not only the participation of trypsin in the digestion of food proteins and other biological processes in the human body is gaining an interest, but it is also used for protein analysis and in biomedicine as well as food and biotechnology industries [5, 6]. The inhibition of digestive enzymes is gaining importance in medicinal chemistry for the treatment of diseases such as platelet aggregation disorders, rheumatoid arthritis, pancreatitis, cystic fibrosis, pulmonary emphysema, and asthma [7]. Therefore, the development of new ligands that can act as trypsin inhibitors is of great interest.

[*] E-mail: ameerkhalid1450@gmail.com
[**] E-mail: mr.alosh618@gmail.com
[***] E-mail: abdulrahman.rasheed9827@gmail.com
[****] E-mail: ahmedraheem311@gmail.com
[*****] E-mail: g.shahinyan@ysu.am

A large number of studies have shown that polyphenols interact with digestive enzymes (such as pepsin, trypsin, etc.) in the process of the digestion and absorption by organism, thus affectinf the activity of both polyphenols and digestive enzymes [8, 9]. Polyphenolic compounds (PCs) are in the focus of many researchers due to a number of physiological properties such as antioxidant, antibacterial, antiviral, anti-inflammatory, anti-obesity, antidiabetic activity etc. [10]. Green tea is one of the main sources of PCs, and its leaves are rich in flavonoids, particularly flavanols and its gallic acid derivatives such as (+)-catechin, (–)-epicatechin, (+)-gallocatechin, (–)-epicatechin gallate, (–)-epigallocatechin, and (–)-epigallocatechin gallate [11–13] (Fig. 1). The inhibition of trypsin by PCs extracted from green tea is extensively studied, whereas the inhibition of digestive enzymes by PCs derived from mountainous herbal infusions remains unexamined.

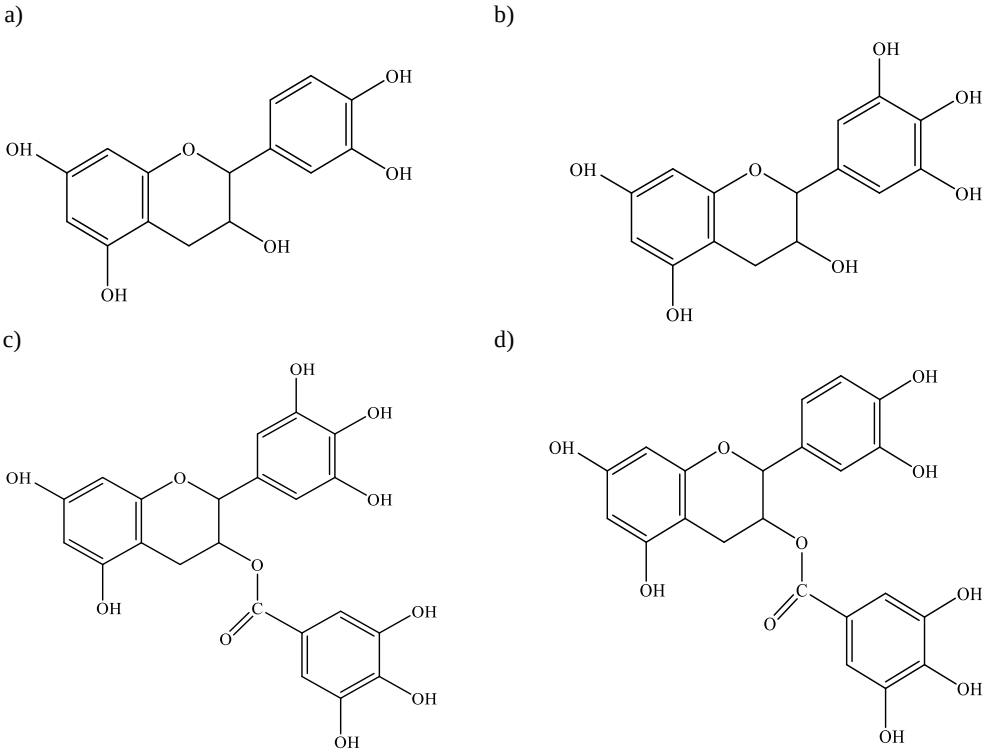

Fig. 1. Structures of polyphenolic compounds present in green tea a) epicatechin; b) epigallocatechin; c) epigallocatechin-3-gallate; d) epicatechin-3-gallate.

Various physiological effects of herbal infusions are well known. Particularly, thyme infusion can be used at acute and chronic respiratory diseases, it has germicidal, anti-inflammatory, sedative and analgesic effects. It can be used as a diuretic and antihypertensive agent [14–16]. Peppermint leaves have anti-inflammatory, analgesic, sedative, as well as stimulant and mood increasing activities [17–19]. Both thyme and peppermint are widely used in many countries as a food and in traditional medicine for the treatment of digestive disorders and nervous system actions. Therefore, the inhibition of digestive enzyme trypsin by PCs derived from thyme and peppermint infusions gains biomedical significance.

Various conditions such as cosolvents, pH and temperature can modify the mechanism of interactions between digestive enzyme and PCs [20–22]. Several studies report that an organic solvent decreases the flexibility of the enzyme and reduces its activity [23–26]. However, it was found that enzyme activity is not always associated to its flexibility; the penetration of an organic solvent into the active site of enzymes may play a significant role in lowering enzyme activity [27]. In reality, the difficulty in empirical identification of small conformational alterations generated by organic solvents is mostly due to the complexity of the system composition and protein structure. As a result, the structure and function of enzymes in non-aqueous environments remain a mystery, particularly at the molecular level. On the other hand, it was shown that the change of pH of the media varies the hydrophilicity of PCs, thus alters inhibitory activity of PCs [28]. Therefore, it is necessary to study the effect of organic cosolvents and pH of the media on the interactions between PCs and trypsin, and understand its mechanism that can provide important information, which in its turn will be useful in biomedical field.

UV-Vis absorption spectroscopy is the most common and convenient technique to study the interactions between small molecules with proteins and nucleic acids [29, 30].

In the present work, the complex formation between trypsin and PCs derived from thyme (*Serpylli herba*) and peppermint (*Menthae piperitae folium*) infusions is studied by virtue of UV-Vis absorption spectroscopy. It should be noted that majority of the published studies report the inhibition of protease enzymes as percent of inhibition and IC_{50} values, whereas the values of physicochemical parameters of PCs–enzyme binding are determined by a few researchers [10]. In our study, the binding constant (K_b) and standard Gibbs energy change (ΔG^0) are determined from the absorption spectra. The effect of polarity and H-bonding ability of the media was studied by the use of various polar organic cosolvents such as ethanol, methanol, acetonitrile. The effect of pH of the media on complex formation is also studied by changing pH from acidic (3.01) to basic (10.01). To reveal the effect of H-bonding ability of a cosolvent on trypsin-PCs interactions two types of polar cosolvents – protic alcohols (ethanol and methanol) and aprotic acetonitrile have been used. The variation of both H-bonding ability and pH of the media provides an information concerning conformational changes of trypsin as well as the main types of non-covalent interactions between PCs and trypsin.

Materials and Methods.

Materials. Bovine trypsin was purchased from "Sigma" (USA). Na_2HPO_4, NaH_2PO_4, CH_3COONa and acetic acid (purity 98%) were of analytical grade. Acetonitrile (purity 99.9%), methanol (purity 98%) and ethanol (purity 96%) were purchased from "PanReac AppliChem ITW Reagents". Double distilled and deionized water (conductance less than 2 $\mu S \cdot cm^{-1}$ at 25°C) was used.

Thyme and peppermint were purchased from specialized tea store. The herbs were collected from different mountainous areas of Armenia located at an altitude of 1600 to 2200 *m* above sea level.

Preparation of Samples. The infusions were prepared using aqueous extraction procedure. 1 *g* of each plant was mixed with 30 *mL* of double distilled water and stirred with a glass rod at 80°C for 10 *min*. All samples were filtered and

cooled to room temperature, after which all samples were diluted 20 times (thyme) and 30 times (peppermint).

Trypsin solution ($8.6 \cdot 10^{-6}$ *M*) was prepared in phosphate buffer (pH 7.4) solution. The solution was stored in a refrigerator at a temperature less than 4°C for at least one day to completely dissolve trypsin.

Three organic cosolvents were used: acetonitrile, methanol, and ethanol with various volume ratios of mixed solvents (organic cosolvent : water) – from 1:6 to 1:2. The pH values of buffer solutions for studying thyme infusion–trypsin and peppermint infusion–trypsin interactions were 3.01, 4.91, 8.64, 10.01 and 3.37, 4.51, 5.48, respectively. For basic solutions, a phosphate buffer was used; for acidic media, acetic acid–sodium acetate buffer was used.

Spectroscopic Measurements. Absorption spectra were recorded using BK-UV1800PC spectrophotometer ("BIOBASE", China), quartz cuvettes were used with length 1 *cm*; pH of buffer solutions was measured by Benchtop pH/ORP/Ion Meter 930 ("BIOBASE"). All experiments were carried out at 20°C, three times and the average value was used. All calculations and mathematical issues, as well as tables, were made using MS Excel 2013 program. Origin 8.5 was used to draw the plots and implement the fitting procedure. To draw the structures of polyphenols ChemBioOffice package was used.

Results and Discussion.

The Study of Trypsin–PCs Non-covalent Interactions. Effect of Polar Organic Cosolvents. To study the interaction between trypsin and PCs derived from mountainous herbal infusions, the UV-Vis absorption spectra of thyme infusion and peppermint infusion were recorded, which are shown in Fig. 2.

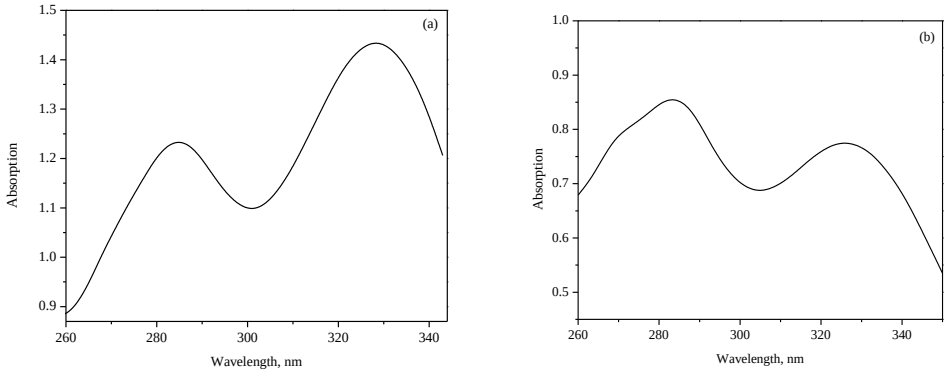

Fig. 2. UV-Vis absorption spectra of thyme (a) and peppermint (b) infusions in water.

The absorption spectra of PCs in herbal infusions show characteristic peaks in the wavelength range from 286 *nm* to 328 *nm*. Fig. 2, a shows that thyme infusion has two peaks at 286 *nm* and 328 *nm*, and the spectrum of peppermint infusion shows two absorption bands at 283 *nm* and 326 *nm* (Fig. 2, b). These absorption bands appear due to the structure of flavonoids, particularly, band I at 250–290 *nm* is due to the benzoyl system, and band II at 300–380 *nm* is due to the cinnamoyl ring [31–33].

Fig. 3 shows the skeleton of flavonoids with corresponding groups. As it is known, the binding of flavonoids to trypsin increases with the increase in the number of hydroxyl groups [10]. On the other hand, the hydroxyl groups responsible for the

antioxidant activity of flavonoids are mainly located in cynnamoyl group, therefore, to study PCs–trypsin interactions we have focused on the change of absorption at 328 *nm* and 326 *nm* for thyme and peppermint infusions, respectively.

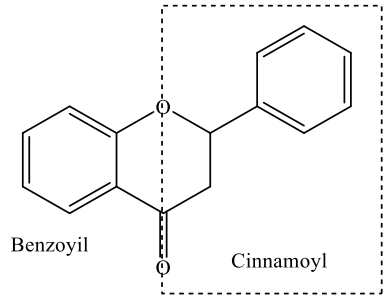

Fig. 3. Figure of flavonoid skeleton.

The addition of trypsin to thyme infusion causes an increase in absorption at 286 *nm* and a decrease in absorption at 328 *nm*. A similar phenomenon is observed for peppermint infusion: absorption increases at 283 *nm* and decreases at 328 *nm*, indicating that there is a ground-state complex formation between trypsin and polyphenols. The inhibitory effects of PCs can be related to non-covalent interactions between digestive enzymes and PCs, which include van der Waals forces, hydrogen bonding, hydrophobic interactions, and electrostatic forces [10].

To calculate the K_b Wolfe–Shimer equation was used [34, 35]:

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} \cdot \frac{1}{K_b[\text{trypsin}]}, \quad (1)$$

where A_0 is absorption without trypsin, A is absorption in the presence of trypsin, ε_G and ε_{H-G} are corresponding molar absorption coefficients in $M^{-1} \cdot cm^{-1}$.

To reveal the effect of H-bonding ability of solvent on trypsin–PCs interaction three organic polar cosolvents were used: protic ethanol and methanol, and aprotic acetonitrile. Using different volume ratios of organic cosolvent–water mixtures, the absorption values at abovementioned two wavelengths for each herbal infusion were measured. Fig. 4 shows the dependences of $A_0/(A–A_0)$ versus 1/[tripsin] in water, and in aqueous solutions of ethanol (a) and acetonitrile (b) for PCs derived from thyme infusion, as well as in water, in aqueous solutions of ethanol (c) and methanol (d) for PCs derived from peppermint infusion. The plots of $A_0/(A–A_0)$ versus 1/[trypsin] show the linear dependence (Fig. 4).

From the slope and intercept the binding constants K_b were calculated. From the values of K_b standard Gibbs energy change can be calculated according to the following equation:

$$\Delta G^0 = -R\,T\,\ln K_b,$$

where R is the gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$), T is the temperature, K. The values of K_b and ΔG^0 change are given in Tab. 1. From the Tab. 1 it can be seen that both thyme and peppermint infusions have strong inhibitory effect on trypsin. Moreover, in an aqueous solution the values of K_b of trypsin–PCs interaction, where PCs are derived from thyme infusion, are larger than that of PCs derived from peppermint infusion, and both are higher than the values reported in [1] for oxyresveratrol and piceatannol. The observed result is not unexpected, as in our study we have a mixture

of different PCs. The comparison of K_b values for PCs derived from thyme and peppermint infusions shows that polyphenols obtained from thyme infusion exhibit stronger inhibition activity compared to peppermint infusion. The value of K_b for PCs obtained from thyme infusion is about six times higher than that of PCs obtained from peppermint infusion, probably due to the concentration of PCs in each infusion.

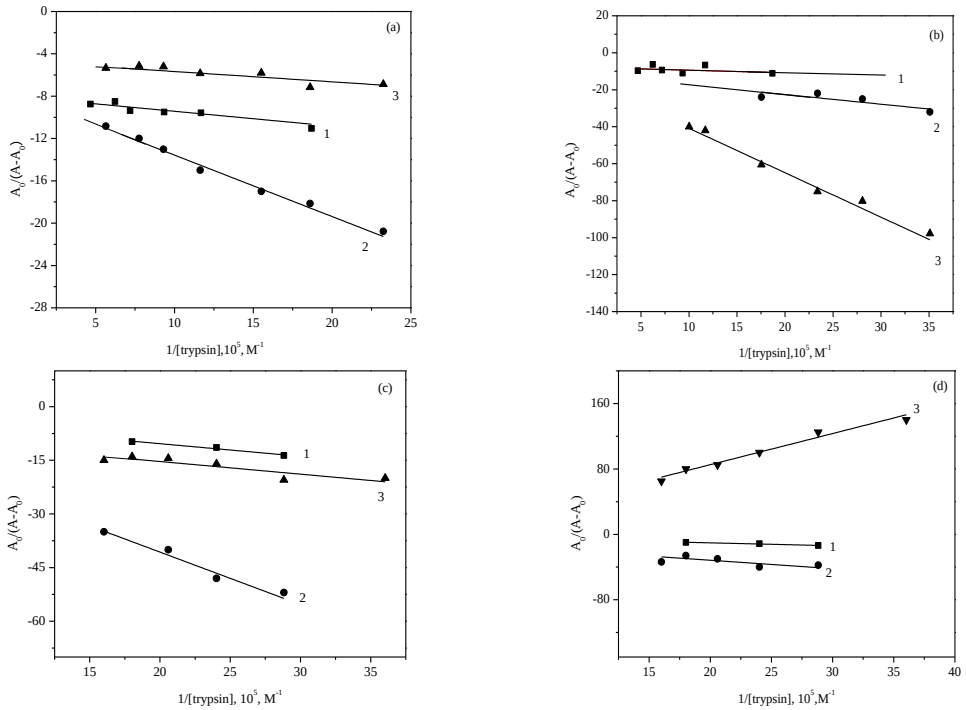

Fig. 4. Dependence of $A_0/(A–A_0)$ on 1/[trypsin] in water and aqueous solutions of ethanol (a) and acetonitrile (b) for PCs derived from thyme infusion, and in water and aqueous solutions of ethanol (c) and methanol (d) for PCs derived from peppermint infusion. Mixed solvents are:
a) 1 – water, 2 – 1 ethanol / 4 water (v/v), 3 – 1 ethanol / 2 water (v/v);
b) 1 – water, 2 – 1 acetonitrile / 4 water (v/v), 3 –1 acetonitrile / 2 water (v/v);
c) 1 – water, 2 – 1 ethanol / 4 water (v/v), 3 – 1 ethanol /2.3 water (v/v);
d) 1 – water, 2 – 1 methanol / 6 water (v/v), 3 – 1 methanol / 2.3 water (v/v).

Tab. 1 shows that the addition of organic cosolvents to PCs–trypsin system causes a decrease in the binding constant, however it should be noted that further addition of ethanol causes an increase in K_b in both cases. On the other hand, with addition of non protic acetonitrile, as well as protic methanol the value of K_b decreases. It can be suggested that the organic solvent alters the structure and dynamics of enzyme, as well as the distribution of the solvent and the hydration process. With addition of organic cosolvent, the enzyme becomes more compact and less native-like compared to an aqueous solution causing the decrease in the availability of the active site. However, further addition of ethanol causes an increase in K_b due to the strong water–ethanol interaction, which results in the displacement of solvent molecules from the active site and thus improves the stability of H-bond

network. On the other hand, the comparison of the effect of a protic solvent such as ethanol and an aprotic solvent such as acetonitrile indicates that hydrogen bonding plays a crucial role in the mechanism of binding of polyphenols to trypsin. Moreover, the change of polarity of the media has considerable effect on electrostatic interactions, which are also significant in PCs–trypsin interaction.

Table 1

Binding constant and standard Gibbs energy change of PCs–trypsin interaction in water and aqueous solutions of ethanol, methanol and acetonitrile

Media (v/v)	K_b, 10^6, M^{-1}	ΔG^0, *kJ/mol*
	thyme infusion	
water	5.723	–37.92
1 ethanol / 4 water	1.337	–34.38
1 ethanol / 2 water	4.827	–37.51
1 acetonitrile /4 water	2.312	–35.71
1 acetonitrile / 2 water	0.699	–32.79
	peppermint infusion	
water	0.932	–33.50
1 ethanol / 4 water	0.773	–33.04
1 ethanol / 2.3 water	2.338	–35.74
1 methanol / 6 water	1.098	–33.89
1 methanol / 2.3 water	0.236	–30.15

From the comparison of K_b values obtained in aqueous solutions of ethanol and methanol for PCs derived from peppermint infusion it can be seen that in methanol K_b decreases about 10 times. As it is known, methanol is a stronger H-bond donor as well as more polar than ethanol. Therefore, it can be suggested that addition of methanol causes the competitive interactions, mainly the formation of hydrogen bonds with the active site of trypsin resulting in the weakening of H-bonds between the active site and polyphenols. In addition, the electrostatic interactions are also weakened with the addition of methanol compared to ethanol. To exclude the effect of solvents on the absorption spectra of thyme or peppermint infusion, the absorbance of both infusions in corresponding mixed solvents in the absence of trypsin were measured at 328 *nm* and 326 *nm*, respectively (data not shown). The addition of acetonitrile has no effect on the absorption of PCs, whereas with addition of ethanol and methanol absorption slightly increases. The latter may be connected with the change of polarity of the media resulting in an increase of extinction coefficient. However, in our measurement the mixture of herbal infusion, water and corresponding amount of polar cosolvent was used as a reference, therefore, this increase can be neglected in discussion of trypsin–PCs interaction.

Effect of pH of the Media on PCs–Trypsin Interaction. In this study, the effect of pH on PCs–trypsin interaction is discussed in both acidic and basic media. For thyme infusion–trypsin interaction we have changed pH from 3.01 to 10.01. The addition of trypsin to thyme infusion at different values of pH causes changes of absorption at both 286 *nm* and 328 *nm*. It is noteworthy that in acidic solutions at pH 3.01 and pH 4.91 with the addition of trypsin the absorption at both wavelenghts

decreases, whereas in basic solutions (pH 8.64 and pH 10.01) the absorption increases at both wavelenghts. To calculate the binding constant the above described method was used. In Fig. 5 the dependences of $A_0/(A–A_0)$ versus 1/[trypsin] in acidic media (a) and basic media (b) are given along with aqueous solution for comparison, where a phosphate buffer with pH 7.4 was used.

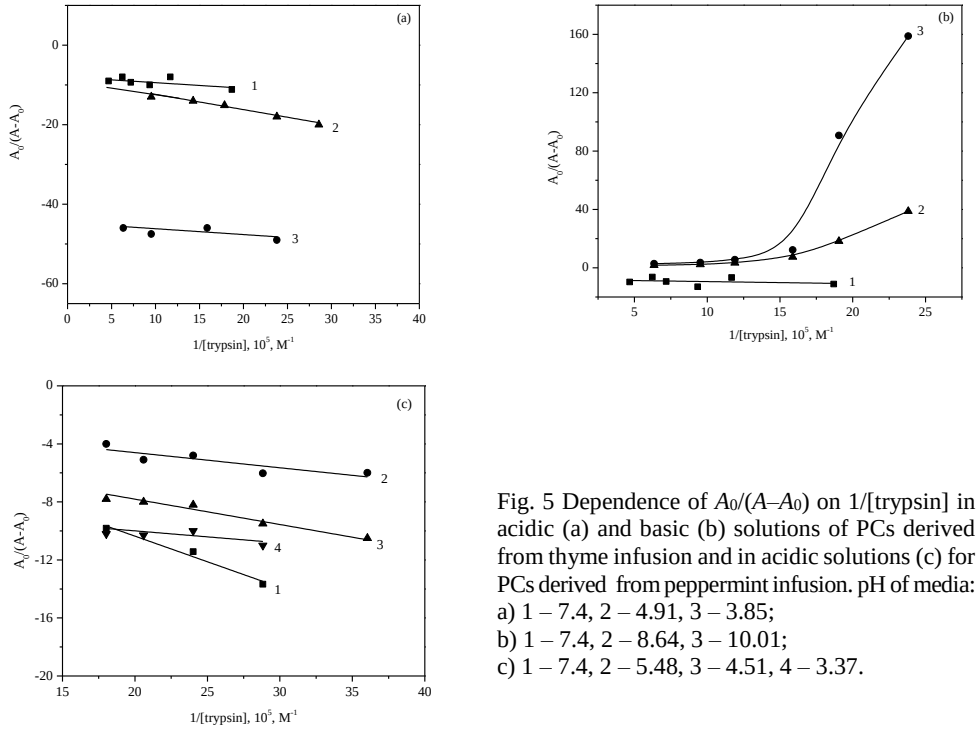

Fig. 5 Dependence of $A_0/(A–A_0)$ on 1/[trypsin] in acidic (a) and basic (b) solutions of PCs derived from thyme infusion and in acidic solutions (c) for PCs derived from peppermint infusion. pH of media:
a) 1 – 7.4, 2 – 4.91, 3 – 3.85;
b) 1 – 7.4, 2 – 8.64, 3 – 10.01;
c) 1 – 7.4, 2 – 5.48, 3 – 4.51, 4 – 3.37.

From Fig. 5 (a and b) it can be seen that the dependence is linear in an acidic solution, whereas in basic solutions the plots are non-linear, which indicates that Wolfe–Shimer equation cannot be used for K_b calculations in basic media. The values of K_b along with ΔG^0 at pH 3.01 and pH 4.91 are given in Tab. 2, which shows that the binding constant increases with decreasing pH. K_b increases by about 10 times, indicating that the activity of trypsin increases sharply in an acidic solution. This may be related to the conformational changes of trypsin resulting in higher availability of the active site for the ligand. On the other hand, it is known that trypsin contains His, Asp, and Ser residues in its binding site [10]. The increase in H^+ concentration causes the protonation of His residue, as a result His residue can interact with the acidic groups of PCs by electrostatic interactions. In the case of basic solutions, the dependence is not linear, and K_b cannot be calculated using the same method. This result is not unexpected, as it is well known that the optimal pH for trypsin activity is 9, and under these conditions trypsin becomes the most stable [36]. In basic media, conformational changes cause a decrease in the availability of the binding site for the ligand and, as a result, complex formation does not occur properly. To observe the effect of pH on absorption of thyme infusion, we measured the absorbance of thyme infusion at 286 *nm* and 328 *nm* in buffer solutions without

trypsin (data not shown). The results show that absorption slightly changes in an acidic solution, whereas in basic solution the values decrease significantly. It can be explained by the deprotonation of both carboxylic groups and hydroxyl groups of PCs. As a result, the distribution of electron density is changed.

As the acidic media strongly affects the activation of trypsin, to study the interactions between trypsin and PCs extracted from peppermint leaves, we have focused on acidic media using acetic acid–sodium acetate buffer and the values of pH were changed from 3.37 to 5.48. Using the absorption values at 326 *nm*, $A_0/(A–A_0)$ and 1/[trypsin] were calculated, and the plots are given in Fig. 5, c. It can be seen that at pH values from 3.37 to aqueous solution (phosphate buffer with pH 7.4) the plots are linear, and using intercept and slope the values of K_b and ΔG^0 were calculated. The values are given in Tab. 2.

Table 2

Binding constant and standard Gibbs energy change of PCs–trypsin interaction in water and acidic solutions

Media	K_b, 10^6, M^{-1}	ΔG^0, *kJ/mol*
	thyme infusion	
water (pH 7.4)	5.723	–37.92
pH 4.91	2.218	–35.61
pH 3.01	30.386	–41.99
	peppermint infusion	
water (pH 7.4)	0.932	–33.50
pH 5.48	2.401	–35.81
pH 4.51	2.466	–35.87
pH 3.37	10.085	–39.30

It can be seen from the Tab. 2, with an increase in acidity K_b of trypsin with PCs sharply increases similar to the results obtained for thyme infusion. Moreover, from a neutral solution to pH 3.37 K_b increases almost 10 times. This result in not unexpected, as with the increase in acidity the activity of trypsin increases.

From Tabs. 1 and 2 it can be seen that the values of ΔG^0 are negative indicating that complex formation between trypsin and PCs derived both from thyme infusion and peppermint infusion in water, aqueous solutions of ethanol, methanol, acetonitrile as well as in acidic media is spontaneous.

Conclusion. Using UV-Vis absorption spectroscopy, binding constant and standard Gibbs energy change for the interactions between digestive enzyme trypsin and polyphenolic compounds were determined. A mixture of polyphenolic compounds were derived from mountainous herbal infusions in particular thyme infusion and peppermint infusion that were collected from mountainous areas of Armenia. The obtained results show that PCs derived from infusions of both herbs have significant inhibitory activity. Moreover, the effect of thyme infusion is stronger compared to peppermint infusion. To reveal the mechanism of interaction, the effect of polarity and H-bond ability of solvent on complex formation using both aprotic (such as acetonitrile) and H-donor (such as ethanol and methanol) organic cosolvents have been studied. The obtained results show that the main driving forces in these

interactions are H-bonds and electrostatic interactions due to a significant change of the binding constant in different mixed solvents. The effect of pH of the media was also investigated, and it was revealed that inhibition of trypsin by polyphenols is maximal in acidic solutions. Moreover, in basic solutions conformational changes of the enzyme cause the restriction of the availability of the active site for ligands.

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Ա. Խ. ՋԱՄԱԼ, Ա. Ք. ՄՈՒՀԱՄԵԴ, Ա. Հ. ՌԱՇԻԴ,
Ա. Ռ. ԱԼ-ՌՈՒԲԱՎԻ, Գ. Ա. ՇԱՀԻՆՅԱՆ

ԲՈՒՍԱԿԱՆ ԹՈՒՐՄԵՐԸ՝ ՈՐՊԵՍ ՏՐԻՊՍԻՆԻ ԱԿՏԻՎՈՒԹՅԱՆ ԻՆՀԻԲԻՏՈՐՆԵՐ ՋՐՈՒՄ ԵՎ ԲԵՎԵՌԱՅԻՆ ՕՐԳԱՆԱԿԱՆ ԼՈՒԾԻՉ–ՋՈՒՐ ՀԱՄԱԿԱՐԳԵՐՈՒՄ

Էլեկտրոնային կլանման սպեկտրոսկոպիայի մեթոդով ուսումնասիրվել է 293.15 *K* ջերմաստիճանում մարսողական ֆերմենտ տրիպսինի ինհիբիցումը լեռնային բուսական թուրմերից ստացված պոլիֆենոլային միացություններով, ինչպես նաև միջավայրի բևեռայնության և թթվայնության ազդեցությունն այդ պրոցեսի վրա: Լեռնային բույսերի (ուրց, դաղձ) թուրմերը լայնորեն հայտնի են՝ շնորհիվ իրենց կենսաբժշկական նշանակության և կիրառության՝ որպես ըմպելիքներ: Հաշվարկվել են կապման հաստատունը (K_b) և ստանդարտ Գիբսի էներգիայի փոփոխությունը (ΔG^0): Ստացված տվյալները ցույց են տալիս, որ բուսական թուրմերից լուծահանված պոլիֆենոլները զգալիորեն ինհիբիցում են տրիպսինի ակտիվությունը: Ավելին, ուրցի ազդեցությունն ավելի զգալի է, քան դաղձինը: Ստացված տվյալները ցույց են տալիս, որ բևեռային օրգանական լուծիչի բևեռայնության և ջրածնական կապ առաջացնելու ունակությունն էական ազդեցություն ունեն K_b-ի արժեքի վրա: Վերջինը վկայում է այն մասին, որ այս փոխազդեցությունների հիմնական շարժիչ ուժը ջրածնական կապերը և էլեկտրոստատիկ փոխազդեցություններն են:

А. Х. ДЖАМАЛЬ, А. К. МУХАМЕД, А. Х. РАШИД,
А. Р. АЛЬ-РУББАВИ, Г. А. ШАГИНЯН

ТРАВЯНЫЕ НАСТОИ КАК ИНГИБИТОРЫ АКТИВНОСТИ ТРИПСИНА В ВОДЕ И В СИСТЕМАХ ПОЛЯРНЫЙ ОРГАНИЧЕСКИЙ РАСТВОРИТЕЛЬ–ВОДА

Методом электронной спектроскопии при 293.15 *K* исследовано ингибирование пищеварительного фермента трипсина полифенольными веществами, полученными из настоев альпийских трав, а также влияние полярности и кислотности среды на этот процесс. Настои альпийских трав (тимьяна, мяты перечной) хорошо известны благодаря их биомедицинской ценности и широкому использованию в качестве напитков. Рассчитаны постоянная связывания (K_b) и изменение стандартной энергии Гиббса (ΔG^0). Полученные результаты показывают, что полифенольные вещества, экстрагированные из травяных настоев, значительно ингибируют активность трипсина. Более того, эффект тимьяна значительно сильнее влияния перечной мяты. Результаты показывают, что изменение полярности и способности к водородным связям органического полярного сорастворителя значительно влияют на значения K_b, тем самым указывая на то, что в данных взаимодействиях главной движущей силой являются водородные связи и электростатические взаимодействия.