Iranian Journal of Catalysis 6(5), 2016, 475-480

IRANIAN JOURNAL OF CATALYSIS

Caffeine effect on adenosine deaminase catalysis: A new look at the effect of caffeine on adenosine deaminase activity

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Received 15 November 2015; received in revised form 3 June 2016; accepted 14 July 2016

ABSTRACT

The effect of physiological concentrations of caffeine (purified from Persian tea) on adenosine deaminase (ADA) activity at physiological and pathological concentrations of adenosine (as substrate) in 50 mM Tris-HCl buffer (pH 7.3) at 37°C was investigated, using UV-VIS spectroscopy. ADA exhibited a bi-phasic activity behavior and both phases showed positive cooperativities indicating adenosine has at least two regulatory sites on ADA molecule. We proposed a mechanism for the inhibitory effect of caffeine on ADA catalytic activity by analyzing ADA kinetic behavior at various physiological concentrations of caffeine (10, 30, 70 μM): Caffeine dominantly resulted in decreased catalytic activity of ADA via reducing the positive homotropic effect of adenosine on ADA activity. Surprisingly, it also showed a very weak but significant activatory effect on ADA activity in a concentration-dependent manner. To the best of our knowledge, this is the first report on the dual effect of caffeine on ADA activity.

***Keywords**: Caffeine, Adenosine deaminase, Inhibition, Homotropic effect.*

1. Introduction

Adenosine deaminase (ADA, EC 3.5.4.4) is an important enzyme (bio-catalyst) in purine metabolism that irreversibly catalyzes the hydrolytic deamination of adenosine or deoxyadenosine to inosine or deoxyinosine, respectively [1]. As a glycoprotein, ADA has a polypeptide chain with 311 amino acids and its gene was sequenced in 1984 [2]. Considering the inhibitory role of caffeine in the binding of adenosine molecules to their receptors, this substance can be helpful in tiredness and drowsiness reduction [3]. Furthermore, caffeine leads to the constriction of brain's blood vessels and, hence, can be used to remediate vascular headache pain [4]. Adenosine deaminase is expressed in all tissues. However, the highest activity of this enzyme is observed in thymus and lymphoid tissues (800 IU/mg) while its least activity has been reported to occur in erythrocytes (1 IU/mg) [5].

Regarding the importance of adenosine deaminase in vital processes such as purine catabolism (an in vivo chemical destructive process) [6], T cell reproduction and immune system strengthening [7], struggling in inflammation (via its known substrate adenosine) and some other disorders such as the inflammatory disease of intestine [8], this enzyme has long attracted a great deal of scientific attention.

Moreover, in different methods such as purification as well as the enzyme activity and structure characterization in living systems like mice and bacterium and its activity in body organs [9-12], the effect of different ligands such as caffeine, aspirin and diclofenac, purine drugs, surfactants and salts has been investigated as well. The role of ADA activity in various pathological conditions and some malignant diseases has been extensively studied [8,13]. Adenosine, as the substrate of ADA is a compensable metabolite in all cells, being involved in key processes such as nucleic acid and purine bases synthesis, amino acid metabolism and cell metabolism homeostasis [14-16].

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Caffeine (1, 3, 7-trimethylxanthine) is a purine alkaloid and its main sources include tea, coffee and cocoa beans [17-20]. In addition to its nutritional benefits, caffeine can be also useful in the treatment of a variety of diseases such as high fat level [21], blood pressure [22], cardiac diseases [23], cancer and AIDS (Acquired immune deficiency syndrome) [24,25]. In the body, caffeine interacts with various biological molecules such as adenosine deaminase enzyme, which is a biological catalyst and plays major roles in purine metabolism. Caffeine has been considered as an inhibitor of such processes [26].

There are many worthwhile reports on ADA activity and its inhibition especially by caffeine [27-33]. In this study, the inhibitory effect of caffeine (extracted from Persian tea) on the pure adenosine deaminase activity at various physiological and pathological concentrations of substrate and inhibitor was investigated.

2. Experimental

2.1. Materials and instruments

Adenosine deaminase from calf intestine was obtained from Roche (product No. 10102105001). Adenosine (substrate) and Tris-HCl were both purchased from Sigma. Calcium hydroxide and dichloromethane were obtained from Merck. In this research, enzyme activity was assayed by UV-Vis spectrophotometer (Shimadzu-UV1800). Ultrasonic bath (230/240V-50Hz Italy Soltec Co.) and refractometer (DR201-95, Germany) were also used for experiments [34].

2.2. Extraction and purification of caffeine from Persian tea

Caffeine extraction and purification from Persian tea were performed based on the ultrasound method [34]. The resulting precipitate was purified by recrystallization in ethanol and the desired product was obtained as a white solid. Product quality was confirmed based on its melting point (238°C), refractive index (1.3341) and ^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ= 3.991 (s, 3 H), 3.58 (s, 3 H), 3.403 (s, 3 H) , 7.512 (s) ppm.

2.3. Adenosine deaminase activity assay

ADA activity (19.5×10^{-3} unit) was assessed by measuring the decrease in absorbance at 265 nm due to the conversion of adenosine (0 to 50.8 μM) to inosine (with extinction coefficient of 8400 $M^{-1}.cm^{-1}$) in 50 mM Tris-HCl buffer (pH7.3), at 37°C [35]. Each assay was repeated at least thrice.

2.4. Determination of kinetic parameters of ADA catalytic reaction

V_{max} was determined using maximum measurement of the enzyme activity. n_{H1} and n_{H2} were determined as slopes of linear parts of Hill Plots. $S_{1/2}$ was determined as the cross-point of graph and *x* axis [36].

2.5. The Effect of caffeine on ADA activity

ADA (19.5×10^{-3} units) was incubated with appropriate concentrations of caffeine for 24h. Before 24 h, ADA activity in the presence of caffeine was associated with some kinetic alterations while upon completion of such time period, it was stabilized. Then, the enzyme solution was assayed as mentioned above.

2.6. Evaluating the reversibility of ADA inhibition by caffeine

Two enzyme solutions were measured for the enzymatic activity (50.8 μM adenosine), in the absence or presence of 10 μM caffeine. Measurements were repeated and compared after dilution of solutions (two-fold dilution and 20-min incubation). All enzymatic measurements were repeated three times.

2.7. Statistical analysis

Statistical parameters such as average, standard deviation and P-value were calculated using Excel software. P-value was determined via T-Test.

3. Results and Discussion

3.1. Extraction of Caffeine

The efficiency of caffeine purification from Persian black tea using ultrasonic bath method was 75%. This method is associated with some advantages including shorter extraction times and lower energy consumption in comparison with other procedures such as soxhlet method.

The main problem in extracting caffeine from tea leaves is the presence of impurities including other natural compounds. Tea leaves contain only 3 to 5% caffeine by dry weight.

As impurities, tannins are polyphenolic compounds (having OH on aromatic ring) [34]. Tannins present in tea are water-soluble and, therefore, are extracted along with the caffeine, which complicates the process of caffeine isolation, because low molecular weight tannins are soluble in dichloromethane as well (see Methods). However, when tea leaves are boiled in the presence of bases such as $Ca(OH)_2$, the ester bonds of tannin are cleaved, resulting in the production of glucose and a calcium salt of gallic acid. These very polar compounds will stay in water and will not be

extracted into dichloromethane. The base also converts caffeine molecules (which may be present as salts) to free base and increases the solubility of caffeine in dichloromethane [34].

3.2. Enzymatic analysis

Adenosine deaminase activity in different concentrations of adenosine (substrate) has been kinetically studied [26-27]. Moreover, "product inhibition" (inhibition of enzyme activity by product molecules) has been reported to occur at high adenosine concentrations (about 200 μM), clearly leading to sever reduction of enzymatic activity [28]. In order to avoid product inhibition, we studied ADA activity at lower concentrations of adenosine (at most 50.8 μM) being closer to its physiological concentrations [37,38]. ADA saturation curve (Fig. 1a) shows a bi-phasic shape with a significant increase in ADA activity in the second phase. To verify such bi-phasic behavior, Eadie-Hofstee and Hill plots were evaluated (Figs. 1b and 1c). The special curvature in Eadie-Hofstee diagram not only verified the bi-phasic behavior but also demonstrated that in the first phase, the enzyme displayed a non-Michaelis behavior and showed positive cooperativity or "positive homotropic effect" [39], indicating that the enzyme activity has been intensified in response to the increase in substrate concentration. Another intensification was observed at higher substrate concentrations (phase II).

The two phases are related to two non-catalytic regulatory binding sites on the enzyme surface, which act at appropriate concentrations of the substrate according to their association constants (k_a). Binding of the substrate to both binding sites led to the increase in enzyme activity. Moreover, according to Hill diagram (Fig. 1c), the cooperativity levels (Hill coefficient, n_H) corresponding to phase I and II were n_{H1}=1.46, n_{H2}= 7.38, respectively.

Consequently, based on the results of the mentioned analysis, at different substrate concentrations adenosine deaminase displayed a non-Michaelis bi-phasic behavior. While such non-Michaelis behavior of ADA saturation curve has been previously reported in higher substrate concentrations [24], the present study is the first report on the bi-phasic behavior of adenosine deaminase in physiological and pathological concentrations of adenosine.

3.3. Reversibility of caffeine effect on ADA activity

Consistent with the findings of a previous report [1], we observed that caffeine (10 μM) causes a significant decrease (10.58%, P= 0.013) in ADA activity (Fig. 2).

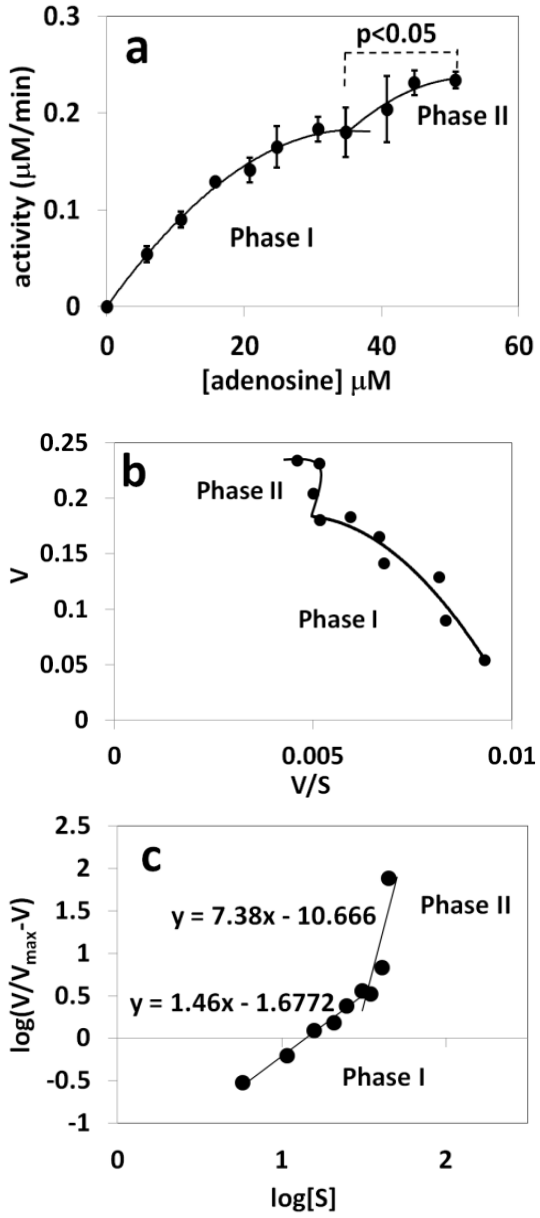

Fig. 1. (a) Adenosine deaminase saturation curve in 50 mM Tris-HCl buffer, pH= 7.3 at 37°C, (b) Eadie-Hofstee plot, (c) Hill Plot.

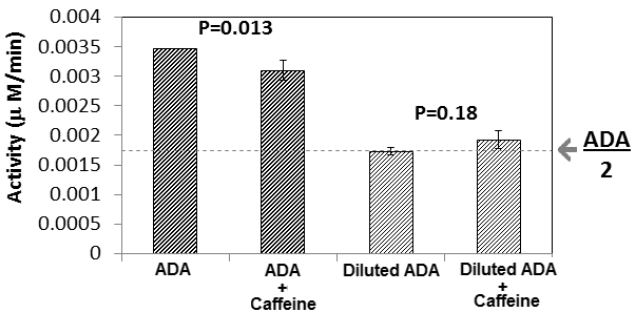

Fig. 2. Reversibility of caffeine effect on ADA activity.

The inhibitory effect was reversible, because after dilution of ADA solution in the absence or presence of caffeine, no significant difference in ADA activity was observed. Reversible inhibitors are classified into three types: competitive, noncompetitive or uncompetitive inhibitors and caffeine acts as an uncompetitive inhibitor for ADA [3].

3.4. Concentration dependency of caffeine activatory effect on ADA activity

Serum caffeine concentration has been reported to be at most 100μM [40]. Therefore, the effect of three different concentrations of caffeine (10, 30 and 70μM) on adenosine deaminase activity was evaluated (Fig. 3a). As illustrated in the Fig. 3, ADA activity significantly decreased in all concentrations of caffeine in comparison with zero concentration of caffeine *i.e.* inhibition. But surprisingly, a little, significant increase was observed with increasing caffeine concentration *i.e.* activation. The results demonstrated that caffeine has a dual effect (both activation and inhibition) on ADA activity, but inhibition is dominant to the activation (Fig. 3a). The activatory effect depended on caffeine concentration. Fig. 3b illustrates the saturation curve of ADA in the absence or presence of the three different concentrations of caffeine.

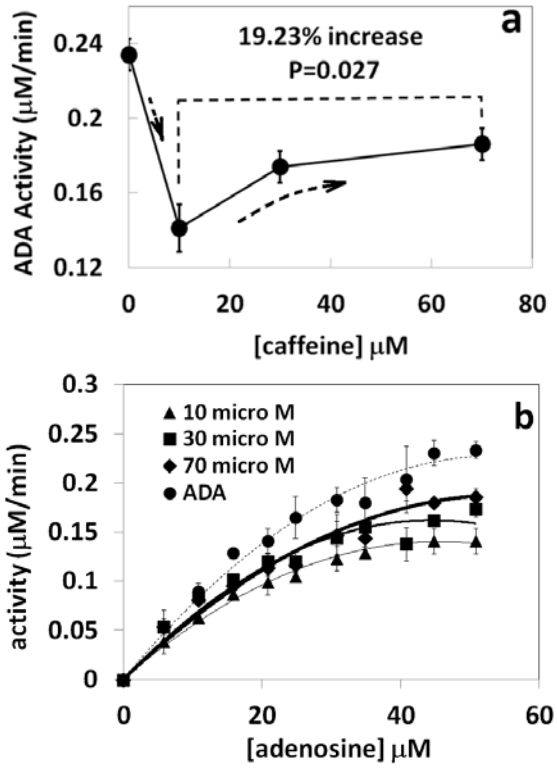

Fig. 3. (a) Activity and (b) saturation curve of adenosine deaminase in the presence of various concentrations of caffeine.

3.5. Kinetic analysis

More careful investigation of saturation curves (Figs. 4a-c) indicated that the bi-phasic behavior was maintained. However, further analysis is required for considering the behavior and quality of positive cooperativity for the two phases in all curves. Eadie-Hofstee plots of mentioned caffeine concentrations confirmed the bi-phasic shape of the plots (Figs. 4d-f). Although caffeine did not change such a bi-phasic shape of Hill plots, it altered the slope of one or both phases called “Hill coefficients” (Figs. 4g-i). Hill coefficients and kinetic parameters of ADA in two phases are compared in Table 1. In addition to this, caffeine exhibited an activatory effect *i.e.* increase in V_{max}) as a result of increase in its concentration, but it was associated with a decrease in Hill coefficients, implying that inhibition and activation mechanisms are different from each other.

The Hill coefficient of the first phase (n_{H1}= 1) in the presence of 10 μM caffeine displayed the Michaelis kinetics and, therefore, the value of $S_{1/2}$ was named K_m. Fig. 5 and Table 1 show the alterations in Hill coefficients as well. The observations were indicative of the mechanism of caffeine inhibitory effect on ADA activity by decreasing the intensity of positive cooperativity of its substrate (homotropic behavior).

4. Conclusion

In this research, caffeine was extracted and purified from Persian tea using the ultrasound method. According to our results, kinetic behavior of different ADA concentrations was bi-phasic. Both phases exhibited positive cooperativity (positive homotropic effect), implying that adenosine has at least two regulatory sites on ADA molecule. The intensity of cooperativity may be affected by caffeine. Studying ADA kinetics in the presence of various physiological concentrations of caffeine (10, 30, 70 μM) demonstrated that caffeine reduces ADA catalytic activity (inhibitory effect) by decreasing the positive homotropic effect of adenosine (substrate) on ADA activity. Despite the inhibitory effect of caffeine, it surprisingly showed a weak activatory effect on ADA activity in a concentration-dependent manner. Our knowledge on the inhibition quality or its mechanism will be helpful to patients being treated with caffeine-containing foods and drugs.

Acknowledgements

The financial support by the University of Kashan is gratefully acknowledged. This study was taken from the MSc thesis of Majid Ghasemi.

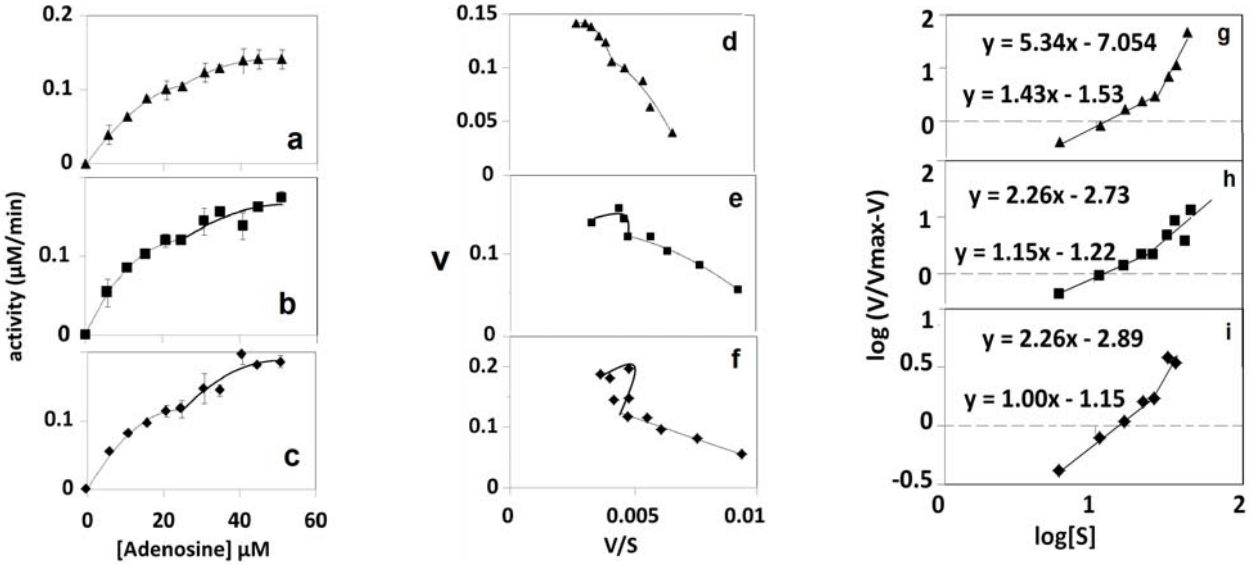

Fig. 4. Saturation curve (a-c), Eadie-Hofstee plot (d-f) and Hill plot (g-i) of ADA in the presence of caffeine (top: 10 μM, middle: 30 μM and bottom: 70 μM).

Table 1. Kinetic parameters of ADA activity in the presence of various concentrations of caffeine.

[Caffeine] μM	n_{H1}	n_{H2}	$S_{1/2}$ (μM)	V_{max} (μM /min)
0	1.46	7.38	14.0	$19.5x10^{-3}$
10	1.43	5.34	11.7	$11.7\ x10^{-3}$
30	1.15	2.26	11.5	$14.5\ x10^{-3}$
70	1	2.26	14.1 (=K_m)	$15.5\ x10^{-3}$

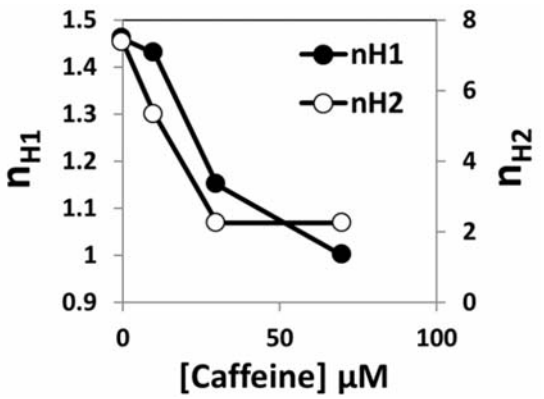

Fig. 5. Hill coefficients of ADA activity as a function of caffeine concentration.

References

[1] R. Hirchhorn, H. Ratech, Isozymes Curr. Top. Biol. Med. Res. 4 (1980) 131-157.

[2] P.E. Doddona, D.S. Schewach, W.N. Kelly, P. Argos, A.F. Markham, S.H. Orkin, J. Biol. Chem. 259 (1984) 12101-12106.

[3] W.F. Schwindinger, L.J. Mihalcik, K.E. Giger, K.S. Betz, A.M. Stauffer, J. Linden, D. Herve, J. Robishaw, J. Biol. Chem. 285 (2010) 29787-29796.

[4] D.A. Pelligrino, H.L. Xu, F. Vetri, J. Alzheimers Dis. 20 (2010) S51-S62.

[5] A. Adams, R.A. Harkness, Clin. Exp. Immunol. 26 (1976) 647-649.

[6] G.C. Mills, F.C. Schmalstieg, K.B. Trimmer, A.S. Goldman, R.M. Goldblum, Proc. Natl. Acad. Sci. 73 (1976) 2867-2871.

[7] C. Herrera, V. Casado, F. Ciruela, P. Schofield, J. Mallol, C. Lluis, R. Franco, Mol. Pharmacol. 59 (2001) 127-134.

[8] L. Antonioli, M. Fornai, R. Colucci, N. Ghisu, F. Da Settimo, G. Natale, O. Kastsiuchenka, E. Duranti, A. Virdis, C. Vassalle, C. La Motta, L. Mugnaini, M.C. Breschi, C. Blandizzi, M. del Taca, J. Pharmacol. Exp. Ther. 322 (2007) 435-442.

[9] L.S. Singh, R. Sharma, Mol. Cell Biochem. 204 (2000) 127-134.

[10] S. Pornbanlualap, P. Chalopagorn, Protein Expression Purif. 78 (2011) 167-173.

[11] K. Kathiresan, K. Saravanakumar, S.K. Sahu, M. Sivasankaran, 3 Biotech. 4 (2013) 235-239.
[12] G.J. Peters, A. Oosterhof, J.H. Veerkamp, Biol. Neonate 42 (1982) 195-200.
[13] J. Meier, M.S. Coleman, J.J. Hutton, Br. J. Cancer 33 (1976) 312-319.
[14] E. Dolezelova, M. Zurovec, T. Dolezal, P. Simek, P.J. Bryant, Insect Biochem. Mol. Biol. 35 (2005) 381-389.
[15] K. Kroll, M.K. Kelm, K.F. Bürrig, J. Schrader, Circ. Res. 64 (1989) 1147-1157.
[16] A. Borowiec, K. Lechward, K. Tkacz-Stachowska, A.C. Składanowski, Acta Biochim. Pol. 53 (2006) 269-278.
[17] H. Wang, L. Chen, Y. Xu, Q. Zeng, X. Zhang, Q. Zhao, L. Ding, LWT-Food Sci. Technol. 44 (2011) 1490-1495.
[18] A. Senol, A. Aydin, J. Food Eng. 75 (2006) 565-573.
[19] H. İçen, M. Gürü, J. Supercrit. Fluids 55 (2010) 156-160.
[20] J. Tello, M. Viguera, L. Calvo, J. Supercrit. Fluids 59 (2011) 53-60.
[21] N.R. Phillips, R.J. Havel, J.P. Kane, Arteriosclerosis 1 (1981)13-24.
[22] M.L. Nurminen, L. Niittynen, R. Korpela, H. Vapaatalo, Eur. J. Clin. Nutr. 53 (1999) 831-839.
[23] M.B. Katan, E. Schouten, Am. J. Clin. Nutr. 81 (2005) 539-540.
[24] H.M. Dreher, J. Psychosom. Res. 54 (2003) 191-198.
[25] G. Nunnari, E. Argyris, J. Fang, K.E. Mehlman, R.J. Pomerantz, R. Daniel, Virology 335 (2005) 177-184.
[26] G. Cristalli, S. Costanzi, C. Lambertucci, G. Lupidi, S. Vittori, R. Volpini, E. Camaioni, Med. Res. Rev. 21 (2001)105-128.
[27] G. Ataie, F. Zonoozi, A. Divsalar, A.A. Moosavi-Movahedi**,** A.A. Saboury, S. Safarian, M. Habbibi-Rezaei, L. Gachkar, S. Mardanian, Pejouhandeh J. 10 (2005) 65-72.
[28] A.A. Saboury, A. Divsalar, G.A. Jafari, A.A. Moosavi-Movahedi, M.R. Housaindokht, G.H. Hakimelahi, J. Biochem. Mol. Biol. 35 (2002) 302-305.
[29] A.A. Moosavi-Movahedi, B. Samiee, G.H. Hakimelahi, J. Colloid Interface Sci. 161 (1993) 53-56.
[30] D. Ajloo, A.A. Saboury, N. Haghi-Asli, G. Ataei-Jafarai, A.A. Moosavi-Movahedi, M. Ahmadi, K. Mahnam, S. Namaki, J. Enzyme Inhib. Med. Chem. 22 (2007) 395-406.
[31] S. Alunni, M. Orrù, L. Ottavi, J. Enzyme Inhib. Med. Chem. 23 (2008) 182-189.
[32] A.A. Saboury, A. Divsalar, G. Ataie, M. Amanlou, A.A. Moosavi-Movahedi, G.H. Hakimelahi, Acta Biochim. Pol. 50 (2003) 849-855.
[33] G. Ataie, A.A. Moosavi-Movahedi, A.A. Saboury, G.H. Hakimelahi, J.R. Hwu, S.C. Tsay, Int. J. Biol. Macromol. 27 (2000) 29-33.
[34] T.J. Mason, Chemistry with Ultrasound, 28th Ed., Springer, 1990.
[35] G. Ataie, S. Safarian, A. Divsalar, A.A. Saboury, A.A. Moosavi-Movahedi, B. Ranjbar, G. Cristalli, S. Mardanian, J. Enzyme Inhib. Med. Chem. 19 (2004) 71-78.
[36] A. Levintzki, D.E. Koshland, Proc. Natl. Acad. Sci. 62 (1969) 1121-1128.
[37] G.H. Möser, J. Schrader, A. Deussen, Am. J. Physiol. 256 (1989) 799-806.
[38] M. Claude, L. Marc, V. Xavier, A. Marie-Laure, G. Regis, Crit. Care Med. 28 (2000) 3198-3202.
[39] J.M. Hutzler, T.S. Tracy, Drug Metab. Dispos. 30 (2002) 355-362.
[40] M. Tarnopolsky, C. Cupido, J. Appl. Physiol. 89 (2000) 1719-1724.