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Research Article
Gentiana lutea Extracts and their Constituents as Inhibitors of Synaptosomal Ecto-NTPDase

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Abstract
The extracellular nucleotides act as neurotransmitters and signaling molecules in CNS, binding to a P2X and P2Y receptors. Their concentration regulates a cascade of membrane ecto enzymes, including the ecto-nucleotide triphosphate diphosphohydrolases (E-NTPDases). In many neuropathological conditions, such as neuroinflammatory, epilepsy, depression and migraine, altering of E-NTPDase activity was observed. The objective of this study was to investigate whether *Gentiana lutea* (*G. lutea*) extracts affect E-NTPDase activity and which of their constituents (loganic acid, gentiopicroside, isovitexin, amarogentin and isogentisin) exert inhibitory activity. The constituent's concentration in the extracts was determined by ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Extracts and constituents were tested with E-NTPDase displayed on the rat synaptosomal membrane as well as by molecular docking study. Ethanol water extract (50%, v/v) exerted significant level of inhibition (52%) at concentration of 200 mg mL^{-1}. By inhibition studies with single constituents about 30% inhibition was achieved in any case, thus the model of one substrate acting on two enzymes was used to determine IC_{50} values. Molecular docking study revealed amarogentin, isovitexin and isogentisin dimer as the potent E-NTPDase inhibitors with the binding energies ranging from -9.4 to -10 kcal mol^{-1} versus -8.0 kcal mol^{-1} for ATP. Presence of isogentisin only in ethanol water extracts may explain their better inhibitory acitivities. Findings of this study are useful from the perspective of safety of products based on *G. lutea* extracts, while investigated constituents belong to secoiridoids and xanthones class of compounds could be considered as a source of potential E-NTPDase inhibitors.

Key words: Amarogentin, ecto-NTPDase, *G. lutea*, synaptosomes, isogentisin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gentiana lutea belongs to Gentianaceae family which comprises more than 1600 species spread in the mountain areas of all around the world. The largest genus in the Gentianaceae family is genus Gentiana, containing about 400 species and among them is *G. lutea* L., which grows in the mountains of central and Southern Europe up to the altitude of 2500 m (Franz *et al*., 2005; Struwe *et al*., 2002).

Secoiridoids and xanthones class of compounds which are the main consituents in the *G. lutea* root exhibit numeorus biological activity and in terms of enzyme inhibition some of them such as: Isogentisine, isovitexin, gentiopicroside and amarogentin showed inhibitory effects on monoamine oxidase type A and B, xanthine oxidase, myeloperoxidase (MPO), aldose-reductase and topoisomerase I (Lin *et al*., 2002; Osamu *et al*., 1978; Akileshwari *et al*., 2012; Nastasijevic *et al*., 2012; Ray *et al*., 1996). Also, in the recent study, it was shown that aqueous extract of *G. lutea* and isovitexin block platelet-derived growth factor induced proliferation of rat aortic muscle cells (Kesavan *et al*., 2013). Hepatoprotective effects of secoiridoids, inhibitory activity toward glycogen phoshorylase and cyclooxygenase 1 and 2 were also described (Mihailovic *et al*., 2014; Vaidya *et al*., 2013; Osamu *et al*., 1978; Park *et al*., 2010). However, in few recent studies it was demonstrated analgesic and anxiolytic effects of *Gentiana* species as well as anticonvulsant and sedative effects of *Swertia* species, in which the most abundant components are secoiridoids and xanthones class of compounds (Jia *et al*., 2012; Tovilovic *et al*., 2011). Due to the increasing number of products that contain in their composition *G. lutea* extracts or extract's fractions, it is still interesting to explore its unrevealed biological effects.

One of those important biological effects could be inhibition of ecto-nucleotidase with special emphasis on E-NTPDase family of enzymes (EC 3.6.1.5) (Robson *et al*., 2006; Zimmermann *et al*., 2012). The E-NTPDase are membrane enzymes ubiquitious in animal cells that hydrolyze extracelular nucleotide tri and diphosphates in the presence of millimolar concentrations of Ca^{2+} and Mg^{2+} at extracellular pH between 7 and 8. It is known that eight different E-NTPD genes encode members of the NTPDase protein family, so far. Four of them (NTPDase 1, 2, 3 and 8) are extracellular membrane located enzymes, present in different proportion at the different type of cells (Zimmermann *et al*., 2012; Maliszewski *et al*., 1994). Results based on immunobloting and hybridizations revealed that in mammalian brain NTPDase 1, 2 and 3 are expressed. However, in the synaptosomal fraction isolated from various sources, the predominant ATPase activity is attributed to E-NTPDase 3 based also on immunocytochemical study (Belcher *et al*., 2006).

The E-NTPDase isoform 3 is ouabain insensitive E-NTPDase, with specificity toward ATP that is about 2.5 fold higher than for ADP. Synaptosomal fractions, containing synaptosomal membranes beside triphosphate/diphosphohydrolase, display on their surface other ectonucleotidases, such as ectonucleotide pyrophosphate/phosphodiesterases (E-NPP), Alkaline Phosphatases (AP) and ecto-5′-nucleotidase (Zimmermann, 2001; Nedeljkovic *et al*., 1998; Yegutkin, 2008). Presence of different types of ectonucleotidases on the membranes exposed to synaptic cleft indicates their role in removing of ATP released into synaptic cleft upon exocytosis of synaptic vesicles (Cognato and Bonan, 2010).

There, ATP together and its ending product of hydrolysis, adenosine act as neurotransmitters, exerting their activity via P1 (Adenosine) and P2 (ATP/ADP) receptors. Altering in E-NTPDase activity was observed in many neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's diseases, then neuroinflammatory diseases such as multiple sclerosis and also in some neuropsychiatric disorders. An increased ATP level exhibits cytotoxic damage and neuroinflammation in the brain tissue. Decreased NTPDase activity leads to increased ATP levels and decreases of adenosine concentration in epilepsy. Therefore, any compound that influences to E-NTPDase activity and their gene regulation may be of importance for the treatment of mentioned disorders (Burnstock, 2010; Roszek and Czarnecka, 2015).

Except of nucleoside analogs and some polyoxometalates, the lack of specific E-NTPDase inhibitors is the main obstacle for development of therapeutics that could modulate purinergic signaling (Muller *et al*., 2006; Al Rashida and Iqbal, 2014). Taking into account the lack of suitable inhibitors and recent findings about effects of secoiridoids and xanthones in the brain we decided to examine *G. lutea* extracts as well as its constituents as potential inhibitors of ecto enzymes present at the membrane of synaptosomes.

The extracts were therefore prepared in a way that is used in human nutrition with water and water-ethanol mixtures as extraction solvents. The main objective of this study was to explain whether and how *G. lutea* affect E-NTPDase based on an investigation of enzyme inhibition with single consituents

and their mixtures. For that reasons, beside their *in vitro* screening, the molecular docking study of consituents was performed.

MATERIALS AND METHODS

Chemicals: Gentiopicroside (5-Ethenyl-6-(β-D-glucopyranosyloxy)-5, 6-dihydro-1H, 3H-pyrano[3, 4-c] pyran-1-one), amarogentin (2-4aS-(4aα, 5ß, 6α))-3, 3', 5-Trihydroxy-(1, 1'-biphenyl)-2-carboxylate), isovitexin (6-ß-D-Glucopyranosyl-5, 7-dyxidroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) were purchased from ChromaDex (Irvine, USA). Loganic acid (1α-(β-D-Glucopyranosyloxy)-6α-hydroxy-7α-methyl-1, 4aα, 5, 6, 7, 7aα-hexahydrocyclopenta[c]pyran-4-carboxylic acid) were purchased from Extrasynthese (Genay, France). Structures of *G. lutea* constituents investigated in this study are shown in Fig. 1.

Acetonitrile, 2-propanol and formic acid were HPLC grade, obtained from J.T. Baker (Deventer, Netherlands) while, glacial acids, also HPLC grade obtained from Fisher Scientific (Leicestershire, UK).

Plant material and extracts preparation: *Gentiana lutea* roots were purchased from the Institute of Medicinal Plant Research "Dr Josif Pancic", Belgrade, Serbia. Five grams of grounded Gentiana roots were extracted with ethanol aqueous (75, 50 and 25% v/v) solutions and water in the ratio 1:20 (w/v). Extraction with ethanol aqueous solutions was performed at room temperature for 48 h with occasional shaking. Water extract was prepared from grounded root suspended in water, followed by heating in a boiling water bath for 10 min. After filtering (0.45 mm filter) extracts were concentrated using a rotavapor at 35°C and kept frozen until analysis.

Preparation of SMP and ATPase inhibition assay: Rat Synaptic Plasma Membrane (SPM) fraction was isolated following the previously described procedure (Gray and Whittaker, 1962). This study was approved by an Institutional Ethics Committee. Protein concentration was determined by the method of Markwell using bovine serum albumin as a standard (Markwell *et al*., 1978). Samples of SPM were kept on -70°C until use. Solution used in enzyme assays were prepared in Milli-Q water. The incubation mixture contained 1 mM $MgCl_2$, 50 mM tris-HCl, pH 7.4 and 10 mg SPM in a final volume of 200 mL. At first, the reaction medium containing SPM was pre-incubated for 5 min at 37°C. *Gentiana lutea* extracts and standards were added and incubated for 20 min (unless otherwise indicated). The assay was initiated by adding 0.1 mM ATP. Incubation lasted 10 min at 37°C and reaction was stopped by the addition of 22 mL 3M perchloric acid. The samples were kept on ice for 15 min before assaying and centrifuged at 5000 g for 5 min to eliminate precipitated protein. Supernatant was used for the colorimetric assay: 80 mL aliquots were withdrawn from each of the reaction samples into wells of a 96-well Nunclon™ surface plate (Nunc, Roskilde, Denmark) and mixed with 20 mL of the Malachite Green Reagent. The plate was incubated for 30 min at room temperature with shaking.

The absorbance at 650 nm was determined using Wallac Victor 1420 Multilabel Counter (Perkin-Elmer Life Science, Waltham, MA, USA). A phosphate standard curve was prepared using a set of phosphate standards ranging in

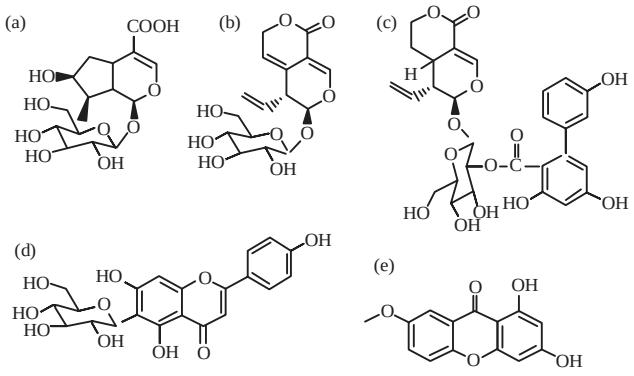


Fig. 1(a-e): Chemical structures of *G. lutea* consituents used in this study, (a) Loganic acid, (b) Gentiopicroside, (c) Amarogentin, (d) Isovitexin and (e) Isogentisin

concentration from 4-40.0 µM, according to the instructions of manufacturer. Experiments were repeated at least three times in duplicate with different enzyme preparations. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding SPM after the reaction was stopped. Enzyme activities were expressed as 10 nmol Pi min^{-1} mg^{-1} of protein. The results are expressed as a mean percent enzyme activity compared to corresponding control value.

Molecular docking study: The crystal structure of *Rattus norvegicus* NTPDase2 used for docking study was downloaded from a Protein Data Bank (PDB code: 4CD3) (Zebisch *et al*., 2014). The water molecules and ligand residues were removed from the structure. The structure of inhibitors (isogenitisin, amarogetin, gentiopicroside, isovitexin and loganic acid) and ATP was obtained by quantum-chemical calculations at B3LYP/6-31G** level, using Gaussian09 program, while dimeric structures of isogentisin (1, 3-dihydroxy-7-methoxyxanthone) were extracted from crystal structure archived in Cambridge Structural Databases (CSD refcode: ABEKOI) (Evans *et al*., 2004; Frisch *et al*., 2009). Docking preparation was done by AutoDockTools program, while the docking study was carried out with AutoDock Vina program (Trott and Olson, 2010). All protein residues were kept rigid and all single ligand bonds were set to be rotational. A grid box, containing the whole protein was used to accommodate the ligand to move freely during docking run.

Chromatography conditions for extracts separation: Waters ACQUITY UPLC system coupled with a Photodiode Array (PDA) detector and Acquity Triple Quadrupole Detector (TQD) with multiprobe source of ionisation including Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) ionisation controlled by MassLynx NT software (version 4.1) was used for qualitative and quantitative analysis. Extracts as well as the gentiopicroside, amarogentin, loganic acid and isovitexin were separated on ACQUITY UPLCTM BEH C18 column (1.7 µm, 100×2.1 mm, Waters, USA) in gradient mode, with the mobile phase consisting of solvent A: Mixture of formic acid and glacial acid (0.9:0.1, v/v in water) and B: Acetonitrile-2-propanol mixture (50:50, v/v) with column temperature of 30°C (Aberham *et al*., 2007). Gradient elution used for separation of extract's component starting with 99% A for 6 min, then from 70-40% A in the next 3 min and from 9-11 min hold at 5% A. Spectra were recorded in the wavelength range from 190-450 nm and chromatographic separation was monitored at 254 nm. The eluent flow rate was 0.3 mL min^{-1}, the injection volume was 5 µL. All extracts were standardized to a concentration of 10 mg mL^{-1} and filtered through 0.22 µm nylon filter (Phenomenex) before injection. Standards were run under the same gradient mode, in concentration range from 0.1-100 µg mL^{-1}. Optimisation of MS conditions was performed by direct infusion of consituents into the mass spectrometer with a syringe pump at flow rate of 20 µL min^{-1}. The source temperature was held at 135°C, desolvation temperature at 350°C, desolvation gas flow was 750 L h^{-1} while cone gas flow was 25 L h^{-1}. The capillary voltage was set at 3.8 kV. In order to find optimal condition for ionisation it was performed tuning of optimal cone voltages, collision energies (using argon as collision gas) and determination of precursor and product iones for all tested constituents. The parameters used for ionization were optimized manually and by IntellyStart in both positive and negative ESI mode. Some of them were already used in the recent study (Lin *et al*., 2015). Acquisition of data were done in MS scan and Multi Reaction Monitoring (MRM) mode, in both ESI positive and negative polarity, with dwell time of 25 m sec as well as with PDA detection.

RESULTS

Qualitative and quantitative analysis of *G. lutea* extracts: In order to perform the qualitative and quntitative analysis of *G. lutea* extracts and to determine the content of its selected constituents (amarogentin, isovitexin, gentiopicroside, loganic acid and isogentisine), UPLC method combined with a PDA and TQD was performed. The UPLC-PDA chromatograms of *G. lutea* water and alcohol water extracts are presented in Fig. 2a. The characteristic absorption spectra of compounds detected in extracts as well as chromatograms and spectra of pure compounds are given in Supplementary material (Fig. S1). Elution method used for UPLC chromatography of extracts combined with acquity BEH column, enabled good separation of *G. lutea* consituents within 11 min of analysis. Mass spectrometry analysis of extracts and constituents was performed in negative ion mode, used for gentiopicroside, isovitexin and amarogentin analysis while the positive ion mode was used in the analysis of loganic acid and identification of isogentisin. Characteristic

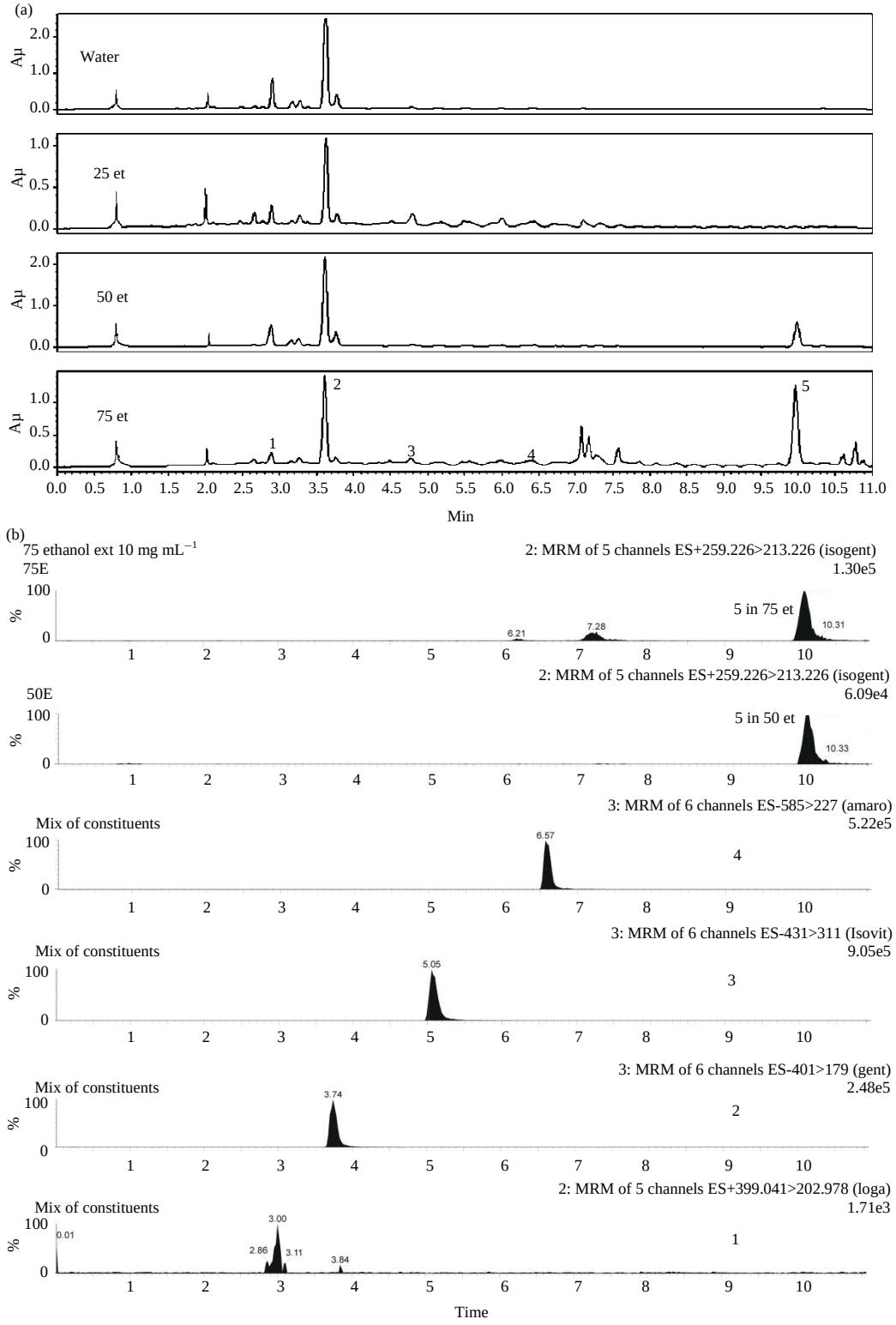


Fig. 2(a-b): (a) UPLC-PDA chromatograms of *G. lutea* extracts (water and 25, 50 and 75% ethanol-water) and (b) UPLC-MS MRM chromatograms, 1: Loganic acid, 2: Gentiopicroside, 3: Isovitexin, 4: Amarogentin and 5: Isogentisin

MRM chromatograms of the constituents used in qualitative and quantitative analysis of extracts are shown in Fig. 2b.

Qualitative analysis was performed by comparison of retention times of standards, their MRM chromatograms and

Table 1: The parameters used in mass spectrometry analysis of *G. lutea* extracts in MRM mode

Compound	Empirical formula	Rt (min)	Detected molecular ions/adducts	MRM m/z*	Cone V (V)**	Coll. E (eV)**	ESI mode
Loganic acid	$C_{16}H_{24}O_{10}$	3.0	$(M+Na)^+$	399.41>202.97	44	22	+
Gentiopicroside	$C_{16}H_{20}O_9$	3.74	$(M-H+HCOOH)^-$	401.11>179	20	12	-
Isovitexin	$C_{21}H_{20}O_{10}$	5.15	(M-H)	431.10>311	45	20	-
Amarogentin	$C_{29}H_{30}O_{13}$	6.79	$(M-H)^-$	585.00>227	60	12	-
Isogentisin	$C_{14}H_{10}O_5$	10.07	$(M+H)^+$	259.00>213	50	35	+

*MRM: Multi reaction monitoring with m/z of parent ion and product ions and **Cone voltage and collision energies

Table 2: Abundance of *G. lutea* constituents in extracts. All experiments were done in triplicates and results are expressed as Mean±SD

*G. lutea constituents	Water	25% ethanol	50% ethanol	75% ethanol
Loganic acid	0.98±0.13	0.30±0.07	0.84±0.02	0.64±0.04
Gentiopicroside	2.53±0.27	0.95±0.09	2.50±0.31	1.65±0.02
Isovitexin	0.004±0.001	0.003±0.001	0.022±0.003	0.011±0.002
Amarogentin	0.014±0.003	0.025±0.002	0.032±0.001	0.041±0.006

*Abundances of constituents are expressed as percentages, calculated on dry matter of *G. lutea* extracts

absorption spectra with corresponding peaks found in chromatograms of extracts. The retention times of selected constituents as well as the parameters used for the mass spectrometry analysis of mentioned compounds in the MRM mode are presented in the Table 1. Total Ion Current (TIC) and MRM chromatograms of standards and extracts are given in Supplementary material (Fig. S2).

Applied conditions used for UPLC-TQD analysis of the extracts in MRM mode gave parent ions which are either protonated and deprotonated molecular ions or sodium and formic acid adducts. Abundance of constituents determined in this study are comparable with those obtained in the previous studies (Aberham *et al*., 2007; Mustafa *et al*., 2015). The abundance of selected *G. lutea* constituents is shown in the Table 2. While, gentiopicroside and loganic acid were found in all extracts as the most abundant constituents, the presence of isogentisin, amarogentin and isovitexin are dependent on extraction procedure.

Thus, the differences between results obtained in this study compared with previous studies could be explained not only by different species (subspecies) used for extract preparation (in this study *G. lutea* ssp., symphyandra) but also with solvents used for extractions, since water and alcohol water mixtures are more polar than 100% methanol. However, the most obvious difference among extracts is the presence of isogentisin, preferebly in alchohol water extracts, especially in 50 and 75% (v/v) extracts (Fig. 2a, b).

Inhibition of E-NTPDase: Before the testing of the interaction between the selected *G. lutea* constituents and E-NTPDase,

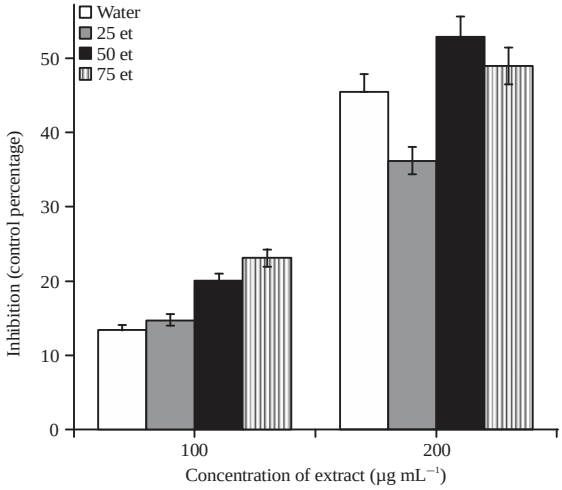


Fig. 3: Inhibition of E-NTPDase in the presence of 100 and 200 µg mL^{-1} *G. lutea* water and ethanol-aqueous (25, 50 and 75%) extracts. The results present the mean values, obtained from two experiments performed in duplicate

the enzyme was exerted to 100 and 200 mg mL^{-1} of water and ethanol (25, 50 and 75%) extracts in the reaction mixture. The working solutions were prepared in water to avoid the enzyme inhibition by organic solvents, i.e., methanol, since in our preliminary experiment its 2% content in water (v/v) inhibits E-NTPDase activity by 24.82%, confirmed also in previous study (Rico *et al*., 2006). The percent of enzyme inhibition in the presence of selected *G. lutea* extracts is shown in Fig. 3.

The results indicated that the significant enzyme inhibition (about 35-50%) was achieved in the presence of 200 mg mL^{-1} of *G. lutea* extracts. In order to elucidate the contribution of each constituent to, alteration of enzyme activity obtained with extracts, E-NTPDase was exposed to the single constituents and their synthetic mixtures. Thus, the inhibitory power of constituents was studied by exerting enzyme to amarogentin, isovitexin and gentiopicroside in the concentration range from 1×10^{-7} to 3×10^{-4} M in the standard enzyme assay. That concentration range was chosen

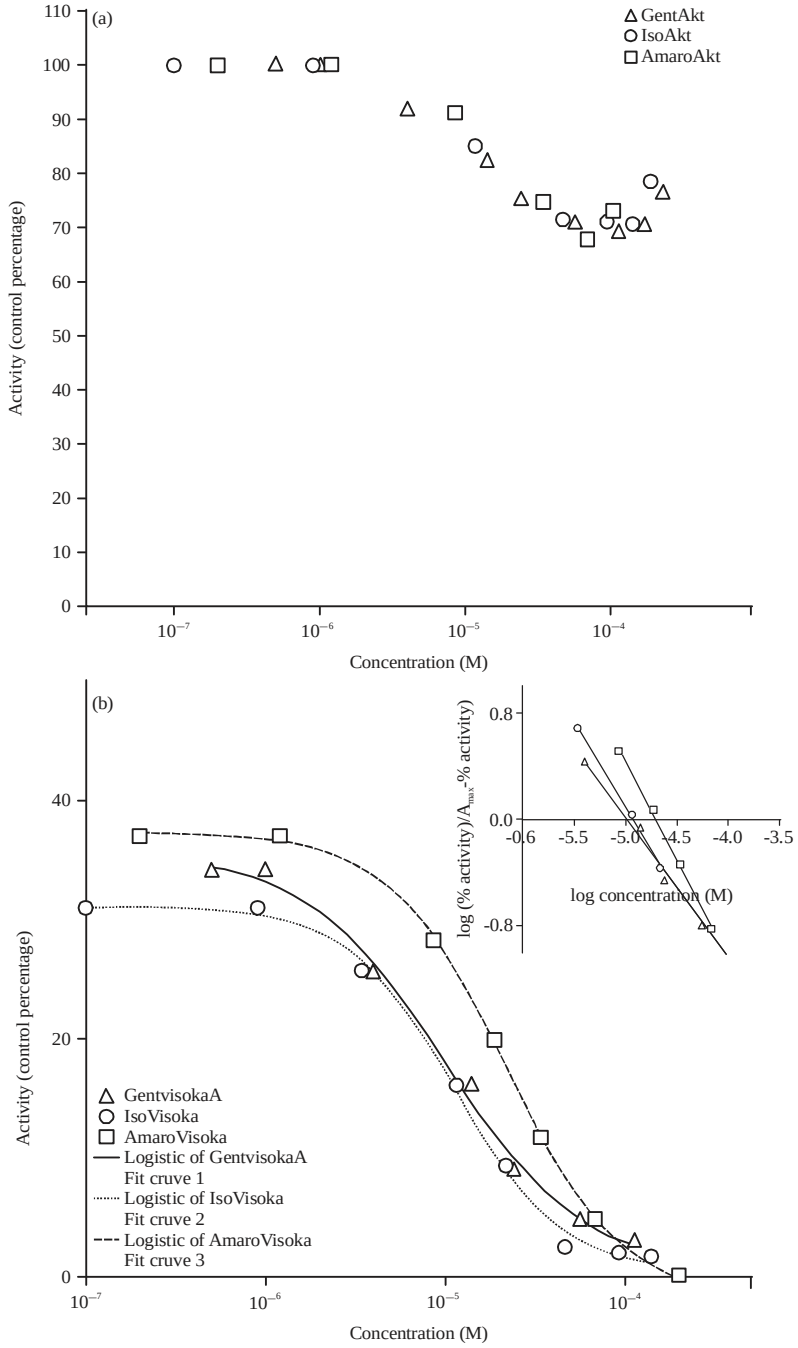


Fig. 4(a-b): (a) E-NTPDase activity in the presence of amarogentin (squares), isovitexin (circles) and gentiopicriside (triangles) in rat synaptic plasma membranes. The values given are the mean of at least three experiments±SEM performed in duplicate and (b) Inhibition curves of high affinity E-NTPDase isoform. Inset: Hill analysis of inhibition curves

based on constituent's abundance in extracts (Table 2) present in 200 µg of extracts. Based on results obtained with enzyme inhibition in a time dependent manner, it was determined that the optimal time of inhibition of the E-NTPDase with all inhibitors, separately was 20 min. The experimental points, that represent the percent of retained activity compared to the control (Fig. 4a) indicated that there was the biphasic response of

enzyme to all selected compounds. Briefly, the certain degree of enzyme activity inhibition (about 35%) was achieved in the concentration range which spanned over 10^{-6} to 10^{-4} M, since by the further increasing of inhibitors concentration the enzymatic activity reminded constant (about 65% of control). Moreover, the inhibition curves overlapped, indicating the similar dose response of the investigated compounds to the enzyme. It is worth to notice that the enzyme activity in the presence of higher inhibitors concentration was not studied because of their low solubility in water.

It is clear from the experimental results that the activity versus constituent's concentration plots in all cases can be represented by the sum of the sigmoid curve and a plateau. In the mathematical analysis of the results presented in Fig. 4a. It was assumed that the mass action principles were fully satisfied and that the plot of the total activity represents the line for "Two enzymes acting on one substrate" (Krstic *et al*., 2004; Vasic *et al*., 1999). The theoretical curves for high affinity enzyme isoforms were obtained by subtracting the values of the plateau from the experimental data and are presented in Fig. 4b. They were fitted by sigmoid function. Moreover, Hill analysis was performed on the high affinity parts of the inhibition curves (Fig. 4b) inset. The values of the Hill coefficient n, determined by Hill analysis of the inhibition curves are summarized in Table 3, together with the IC_{50} values obtained by fitting the constructed inhibition curves with sigmoid function. The Hill analysis always yielded n>1, suggesting the cooperative binding of the inhibitor. Using the calculated IC_{50} values, the total activity was recalculated and presented in Fig. 4a as a solid curve. The results show that an excellent fit of the experimental points was obtained.

Since all of the tested constituents exerted a similar level of inhibition, it was desirable to determine how they inhibit enzyme when they are present together, like in extracts. For this reason, mixtures of mentioned constituents, which contain gentiopicroside, isovitexin and amarogentin in four different combinations were prepared as it is shown in the Fig. 5.

The highest degree of inhibition, about 16% was achieved with the mixture containing gentiopicroside, isovitexin and amarogentin, each in final concentration of 20 μg mL^{-1} (Table 4). All other mixtures, containing two constituents (concentration in the mixtures, 20 μg mL^{-1}) inhibited enzyme to lower extent (10, 5 and 3%). However, inhibition of E-NTPDase achieved with single

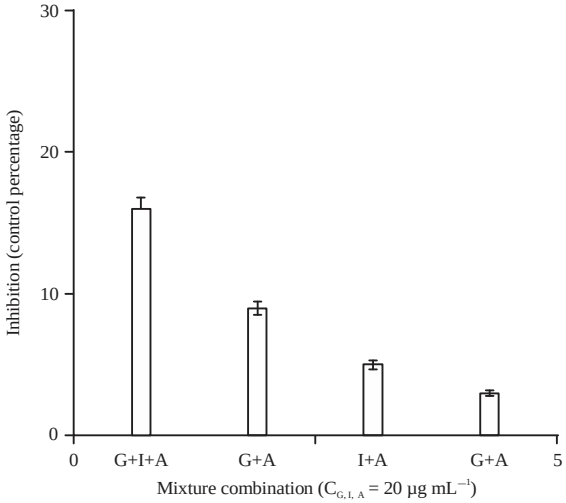


Fig. 5: Inhibition of E-NTPDase with mixtures of constituents (constituent's names in the mixture are indicated by their initial letters). The results present the mean values obtained from two experiments performed in duplicate

Table 3: Parameters of E-NTPDase inhibition induced by amarogentin, isovitexin and gentiopicroside

	Sigmoid curve		Hill analysis		
Compound	IC_{50} (M)	p*	IC_{50} (M)	n**	IC_{50} (μg mL^{-1})
Amarogentin	9.89×10^{-6}	1.12	(1.02×10^{-5})	1.04	5.79
Isovitexin	1.13×10^{-5}	1.45	(1.17×10^{-5})	1.31	4.88
Gentiopicroside	2.15×10^{-5}	1.35	(1.95×10^{-5})	1.48	7.66

*Parameter of sigmoid function and **Hill coefficient

Table 4: E-NTPDase activity in the presence of constituents and their mixtures (constituent's names in the mixture are indicated by their initial letters) All experiments were done in duplicate and results are expressed as Mean±SD

Inhibitor	Concentration (μM)*	E-NTPDase activity (Inhibition control percentage)
Gentiopicroside (G)	56	70.7 (29.3±1.2)
Isovitexin (I)	46	71.5 (28.5±0.8)
Amarogentin (A)	34	74.7 (25.3±1.8)
I (G+I+A)	56+46+34	84.5 (15.5±3.4)
II (G+I)	56+46	90.2 (9.8±0.2)
III (G+A)	56+34	97.0 (3.0±1.2)
IV (I+A)	46+34	94.7 (5.3±0.9)

*C = 20 μg mL^{-1} for each constituents individually and in the mixtures

constituents at the same concentration was 29.30, 28.5 and 25.30% for gentiopicroside, isovitexin and amarogentin, respectively. Comparison of these results with those obtained from the inhibition curves leads to the conclusion that these three constituents exhibit antagonistic effects when they are present in the mixture in the same mass concentration.

Therefore, results obtained with mixtures of constituents cannot explain higher inhibition (more than 50%) of tested extracts. However, the possible explanations could be given according to concentration of constituents in tested extracts (Table 2). Namely, in the inhibition experiments with single constituents, the lowest concentration for all tested constituents was 5 μg mL^{-1} (molar concentration range from 8×10^{-6} to 1.2×10^{-5} M). At that concentration gentiopicroside, isovitexin and amarogentin exerted inhibition of 18.0, 14.9 and 8.7%, respectively. It is obvious that only 200 μg of water and 50% ethanol extract, contain 5 μg mL^{-1} gentiopicroside. Concentration of isovitexin and amarogentin in 200 μg of these extracts are too low to inhibit E-NTPDase.

Results obtained in experiment with single constituents, clearly indicate that there was no E-NTPDase inhibition in the molar concentration range from 10^{-9} to 10^{-7} M. Despite that, extracts in a concentration of 200 μg mL^{-1} inhibit E-NTPDase in the range of 36-52%. That degree of inhibition is obviously the result of synergistic effect of gentiopicroside with other constituents present in *G. lutea* extracts. They include swertiamarin and sweroside, which belongs to secoiridoid class of compounds and isogentisin and gentisin belonging to xanthones class of compounds. Otherwise, isogentisin were found only in 50 and 75% ethanol water extracts. Thus, it could be proposed that isogentisin significantly contribute to the inhibitory acitivity of ethanol water extracts and could be a potentially strong inhibitor of E-NTPDase.

Molecular docking study: Therefore, the molecular docking study with isogenitisin as inhibitor and with ATP as substrates of E-NTPDase was carried out. For this study, E-NTPDase isoform 2 was chosen since it is the most closly related to cell surface-located NTPDase3 (Vorhoff *et al*., 2005). There is no available crystal structure of E-NTPDase3 in the PDB database. The E-NTPDase3 is the predominant isoform of ecto enzymes displayed in rat SPM, followed with isoforms 2 and 1. Analysis of the docking results predicted only one potent binding site for isogentisin on E-NTPDase2 (Fig. 6) and this site overlaps with binding site for ATP on enzyme (the nucleoside binding site). These results indicate on the competitive inhibition of E-NTPDase2 enzyme. Estimated binding energy is slightly greater for ATP (-9.3 kcal mol^{-1}) comparing to isogentisin (-8.0 kcal mol^{-1}). The reason for the energy differences probably is a greater number of hydrogen bonds between ATP and amino-acid residues of enzyme (Gly 47, Ser 48, Ala 123, Gly 124, Gly 204, Ala 205 and Val 434) than the number of hydrogen bonds between isogentisin and enzyme (Ser 48, Thr 122, Gly 204 and Trp 436) (Fig. 6). However, the binding of isogentisin to the active site of the enzyme, despite the lower binding energy than ATP can be explained by smaller size of isogentisin and almost planar structure that enables its greater mobility, compared to ATP molecule.

Docking studies with amarogentin, gentiopicroside, isovitexin and loganic acid as inhibitors of E-NTPDase were also carried out. The results indicate on the competitive inhibition, because the studies showed only one potent binding site for amarogetin, gentiopicroside, isovitexin and loganic acid on E-NTPDase2 (Supplementary material, Fig. S1-S8), which overlaps with binding site for ATP on enzyme. Estimated binding energies (Table 5) are greater for amarogentin (-9.4 kcal mol^{-1}), gentiopicroside (-8.5 kcal mol^{-1}), isovitexin (-9.4 kcal mol^{-1}) and loganic acid (-8.6 kcal mol^{-1}) comparing to isogentisin (-8.0 kcal mol^{-1}). The binding energy differences can be explained by differences in the size of inhibitors (isogentisin, amarogentin, gentiopicroside, isovitexin and loganic acid) and by differences in the nature and number of interactions between inhibitor and amino-acid residues of enzyme. In comparison with isogentisin, amarogentin, gentiopicroside and loganic acid form a greater number of non-covalent interactions with amino-acid residues of enzyme (Supplementary material, Fig. S3-S8). It known that hydrogen bonds make a favorable contribution to protein stability (Pace *et al*., 2014). Figure 6 and 7 illustrate the hydrogen bonds in the most stable cluster of inhibitor and E-NTPDase2 enzyme. One can conclude that there are also a greater number of hydrogen bonds between amino-acid residues of enzyme and amarogentin, gentiopicroside, isovitexin and loganic acid (Fig. 7) than a number of hydrogen bonds between enzyme and isogentisin (Fig. 6). Despite the fact that the binding of amarogetin, gentiopicroside, isovitexin and loganic acid to the active site seems like more sterically hindered than the binding of planar isogentisin

Table 5: Binding energies of isogentisin, amarogetin, gentiopicroside, isovitexin and loganic acid as inhibitors of E-NTPDase and for ATP as a substrate of E-NTPDase

Ligand	Binding energy (kcal mol^{-1})
ATP	-9.3
Isogenitisin	-8.0
Isogenitisin (dimer)	-10.0
Amarogetin	-9.4
Isovitexin	-9.4
Gentiopicroside	-8.5
Loganic acid	-8.6

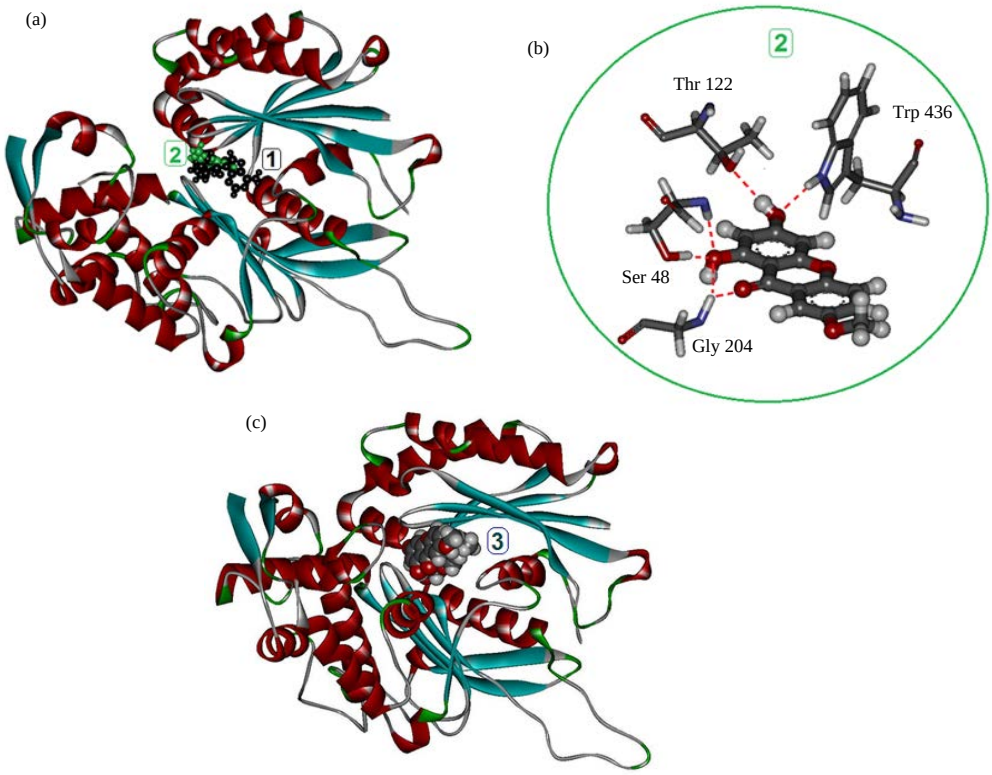


Fig. 6(a-c): (a) Binding sites of ATP, (b) Monomeric structure of isogentisin and (c) Dimeric structure of isogentisin at rat NTPDase2 enzyme. Hydrogen bonds of monomeric isogentisin with Ser 48, Thr 122, Gly 204 and Trp 436 residues are depicted in the middle by red dotted lines

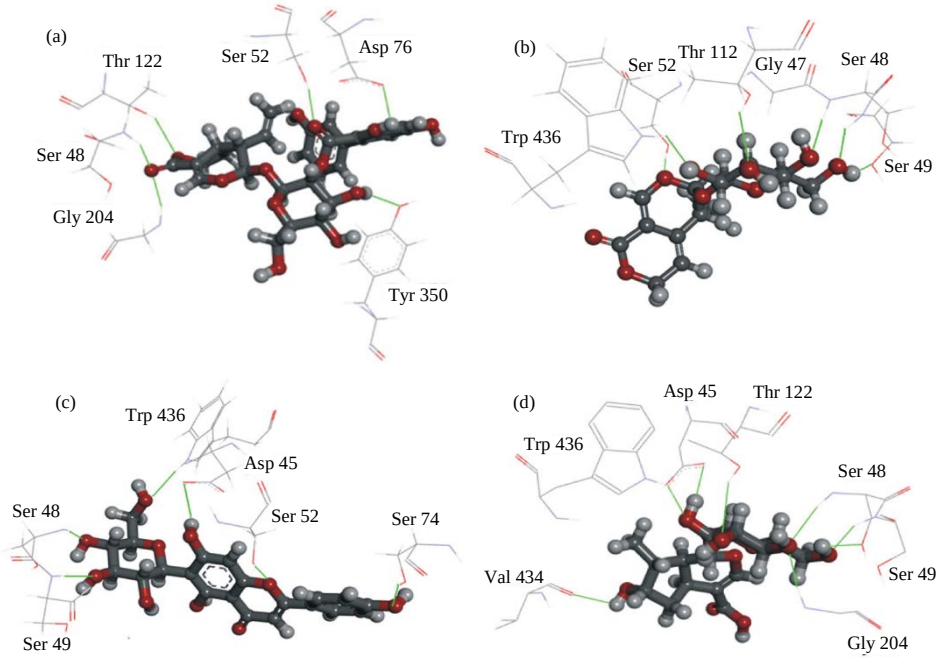


Fig. 7(a-d): Hydrogen bonds of (a) Amarogetin, (b) Gentiopicroside, (c) Isovitexin and (d) loganic acid in most stable cluster of inhibitor and E-NTPDase2 enzyme. Hydrogen bonds are depicted by green line

molecule, the higher flexibility of these three inhibitors (expressed by a larger number of rotatable bonds) is in favor of their stronger binding.

The second possible manner for binding of isogentisin on NTPDase2 was found by analyzing crystal structures archived in the PDB. In both of the two crystal structures (PDB codes: 4CD1 and 4CD3) the sulfoanthraquinone inhibitor PSB-071 (structurally similar to isogentisin) binds as a sandwich of two molecules at the nucleoside binding site (Trott and Olson, 2010).

DISCUSSION

Gentiopicroside is the most abundant secoiridoid constituents in the *G. lutea* herb but its concentration depends on extraction preparation procedure. Thus, the extracts obtained with alcohol water mixtures, where ethanol is present in volume concentration of about 50% or higher, yield the highest abundance of gentiopicroside (Arino *et al*., 1997). However, extraction with boiling water contributes that extraction of gentiopicroside approaches or surpass those obtained with alcohol water mixtures. Also, extraction of loganic acid with water yielding to its highest content in tested extracts. Eventhough amarogentin was proved as the most potent inhibitor of E-NTPDase, its abundance in extract is much lower and its presence could be detected occasionally in *G. lutea* species, varying from 0.03 to more than 0.3%, depending on its origin and root processing method (Azman *et al*., 2014; Aiello *et al*., 2013).

Therefore, it could be proposed that increased inhibitory activity of extracts toward E-NTPDase could be attributted to other constituents. That hypotesis was also supported with E-NTPDase inhibition assay performed with mixtures of constituents. Even present in higher concentration than in extracts, amarogentin, isovitexin and gentiopicroside in mixtures did not achieve inhibition as each extract alone. Other pharmacologically relevant constituents present in the extracts are swertiamarin, sweroside and secoiridoids with similar structure as gentiopicroside. The abundance of these compounds is in the range from 0.2-0.4% for sweroside and swertiamarin and from 0.02-0.11% for gentisin and isogentisin which belong to xanthones class of compounds. There are also present other xanthones such as gentioside, gentisin-1-O- primaveroside. Of other consituents there are volatile oils, in the concentration up to 0.2% and carbohydrates, mono, di and tri saccharides (EMA., 2009). Based on concentration of gentiopicroside in all tested extracts, ranging from 0.9-2.5% and corresponding inhibition of E-NTPDase from 36-52%, it can be concluded that inhibition depends on the increased concentration of gentiopicroside.

However, 75% ethanol water extract contains lower quanitiy of gentiopicroside than water and 50% ethanol water extracts but still inhibits E-NTPDase stronger than water. It is obvious that other compounds present in ethanol water extracts, contribute (probably synergistically) to stronger inhibition of enzyme. Since, all constituents of *G. lutea* did not tested *in vitro*, the molecular docking study was performed with a special emphasis on isogentisin. According to results obtained with extracts it was hypothesized that isogentisin significantly contributes to inhibition of E-NTPDase.

The results of docking study coincide with those obtained experimentally because amarogentin is proved to be most potent inhibitor both in inhibition assay with E-NTPDase and in docking study. Namely, the binding energy of amarogentin is -9.4 kcal mol^{-1}, while IC_{50} = 9.89 μM. Binding energy for isovitexin is the same as for amarogentin but experimentally determined IC_{50} of isovitexin is 11.3 μM. Gentiopicroside binding energy is -8.5 kcal mol^{-1} and thus determined IC_{50} is higher, i.e., 21.5 μM. Loganic acid, which was excluded in our preliminary experiment, because it did not show inhibition at investigated concentration has the binding energy as gentiopicroside. Finally, results of molecular docking study of isogentisin showed the lowest binding energy in comparison with other constituents (-8.0 kcal mol^{-1}). However, isogentisin dimer binds at the same site as isogentisin monomer and ATP (Fig. 6) but with greater binding energy (-10.0 kcal mol^{-1}, Table 5). Better binding of dimeric structure to the active site is important but not decisive for inhibitory activity, because the approach of the dimeric structure to the active site is more sterically hindered than the approach of monomer or of ATP to the active site. However, the binding of isogentisin to the active site of the enzyme, despite the lower binding energy than ATP can be explained by the smaller size of isogentisin and its almost planar structure that enables its greater mobility, compared to the ATP molecule.

Even though, there are two possible explanations about the inhibition of E-NTPDase with isogentisin, its structure indicates that it could be potential inhibitor of this enzyme. Namely, in addition to the structure similarity with sulfoanthraquinone, there are also found

similarity with reactiv blue 2 (sulfonated dyes) structure (Muller *et al*., 2006; Al Rashida and Iqbal, 2014). In the study performed with the ADP induced platelet aggregation, it was shown inhibition of aggregation with xanthones derivates (Rajtar *et al*., 1999). Although platelets contain membrane's E-NTPDase isoformes in different proportion then synaptosomes, that finding supports hypothesis proposed in this study.

When considering other class of compounds as inhibitors, it was shown in previous studies, that nonselective NTPDase inhibitors achieve 25% of inhibition in the concentration range between 17-62 μM for cibacron blue, uniblue and mentioned reactive blue as well as aromatic isothiocyanato sulfonates in the concentration range from 10 to 464 μM (Al Rashida and Iqbal, 2014). In the recent study, which was comprised the screening of 438 structurally diverse drugs it was proposed that compounds which show at least 30% of inhibition at 20 μM concentration should be considered as hit compounds (Fiene *et al*., 2015). In fact compounds that have proven to be potent inhibitors of these enzymes belong to the polyoxometalate class of compounds, with IC_{50} in the range from 1.7-3.4 for 12-tungstosillicid acid and 12-tungstophosphoric acid (Colovic *et al*., 2011). Thus, considering *G. lutea* constituents in any of proposed manner, one can conclude that they are promising candidate for inhibitor development since their IC_{50} is in the range of 10-20 μM. On the other hand, there are no sufficient data about inhibition of E-NTPDase with herbal extract, so far. However, it was found out in a recent study, that six plant extracts, among fifty tested, exert a significant level of inhibition, above 50%, toward E-NTPDase present in the crude enzyme preparation of chicken liver (Ashraf *et al*., 2011). This type of E-NTPDase found in liver, bone and kidney is tissue nonspecific AP with affinity toward various monoesters of phoshoric acid. The difference from those E-NTPDase present in the rat synaptosomal membrane (type 3) is different affinity toward ATP as well as its final products.

Plant extracts exerting inhibitory activity toward liver E-NTPDase belong to Lilliaceae, Asteraceae and Apiaceae family of herbs and all of them inhibited E-NTPDase at the concentration of 125 μg mL^{-1} in the range from 51% to approximately 58%. Among them the best inhibition was achieved with *Asparagus officinalis* methanolic extract, with an inhibition of 58.24% toward E-NTPDase which is comparable with inhibition achieved with *G. lutea* extracts, with an inhibition of 52.89% at concentration of 200 μg mL^{-1} (50% ethanol aqueous). The results obtained in both studies could be of interest, since E-NTPDases expressed in various tissues show different affinity toward ATP and ADP, thus it is quite possible to expect some differences in inhibition (Zimmermann, 2001). For instance, inhibition of E-NTPDase present on human lymphocytes belonging to CD39 family (isoform 1) has pro-immflamatory effects (Chadwick and Frischauf, 1998).

Significance of results obtained using rat's synaptosomal membranes as a model system for investigation of E-NTPDase activity in the presence of *G. lutea* extracts is according to role of ATP in the brain, where it acts as a rapid neurotransmitter. There, ATP binds to the P2X purinoceptor, a ligand-gated ion channel and its action is related to demyelination and remyelination of axons, termination of purinergic signaling and development of neurones. Any variation from its physiological concentrations may cause adverse effects. It was found, in the human actively spiking regions, that the activities of synaptosomal E-NTPDase are decreased. Hence, an additional inhibition of this enzyme, with inhibitors, like herbal preparations, may cause hyperactivity and increased excitability in the epileptic brain (Horvat *et al*., 2006).

CONCLUSION

Results demonstrate that *G. lutea* extracts are potent E-NTPDase inhibitors. The highest level of inhibition (about 50%) was achieved with ethanol water extracts in a concentration of 200 mg mL^{-1}. The constituents shown approximately the same level of inhibition with IC_{50} ranging from 1 to 2×10^{-5} M. The results of molecular docking study are identified gentiopicroside, amarogentin, isovitexin and isogentisin dimer as potential enzyme inhibitors with binding energies slightly greater than for ATP. These results could be useful for the evaluation of the safety of herbal preparations, particularly those belonging to the Gentianaceae family of herbs. Furthermore, compounds related to secoiridoids and xanthones class of compounds could be examined as potential enzyme inhibitors.

ACKNOWLEDGMENTS

The authors would like to thank to the Ministry of Science and Technological Development of the Republic of Serbia, Project No 172023 as well as to the FP7 FUNCFOOD project, Grant agreement no. 245030 for their financial support.

SUPPLEMENTARY MATERIALS

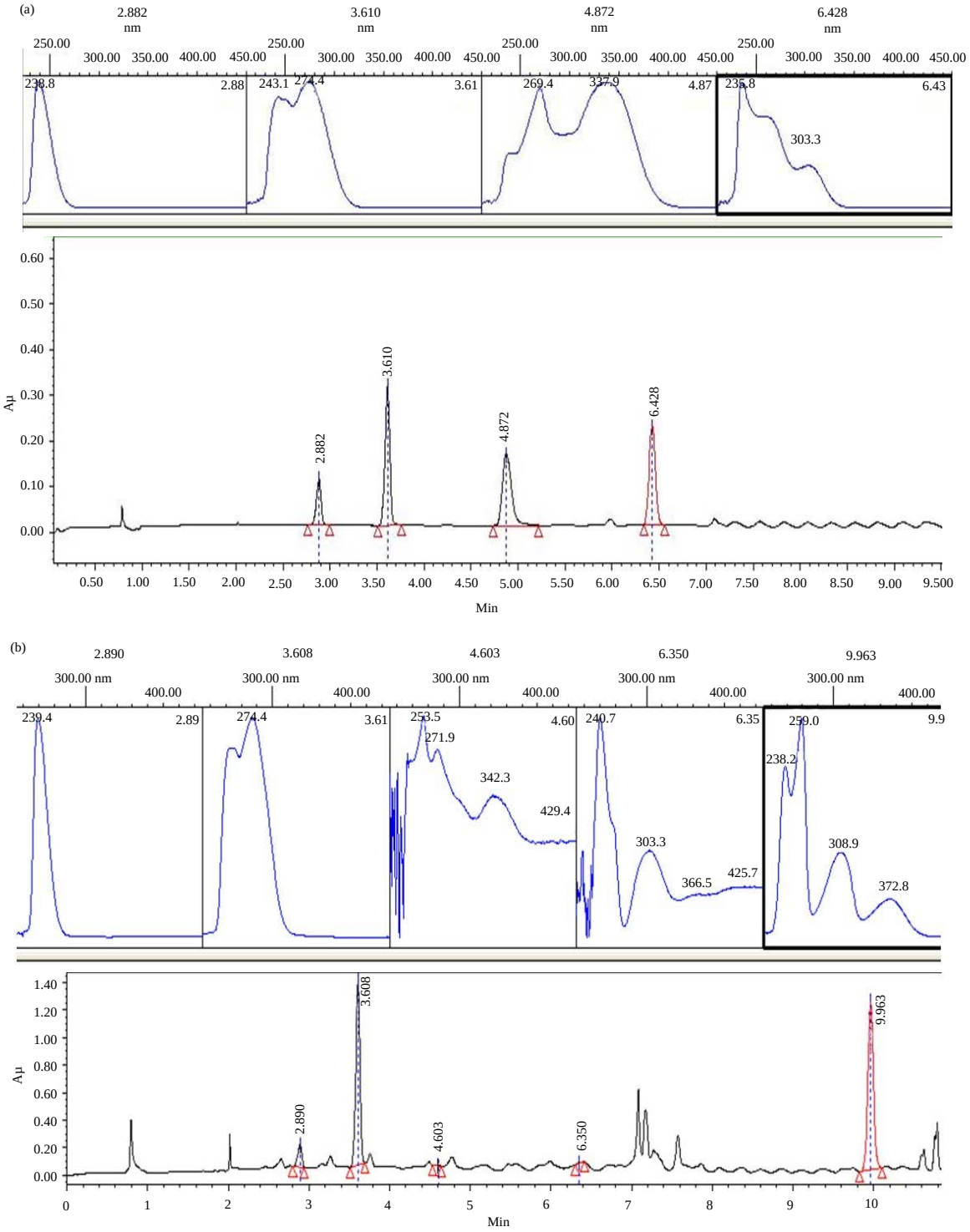


Fig. S1(a-b): (a) UPLC/PDA chromatograms and extracted absorption spectra of *G. lutea* constituent's mixtures (C = 100 μg mL^{-1}) with loganic acid (Rt = 2.88), gentiopicroside (Rt = 3.67), isovitexin (Rt = 4.87) and amarogentin (Rt = 6.43) and (b) Chromatogram of 75% ethanol water extract (C = 10 mg mL^{-1}) and absorption spectra of its constituents with isogentisin at Rt = 9.96 min. Isogentisin (from 50 and 75% ethanol water extract) spectrum with absorption maxima at 238.2, 259.0, 308.9 and 372.8, already confirmed in the previous study (Morimoto *at al.*, 1983)

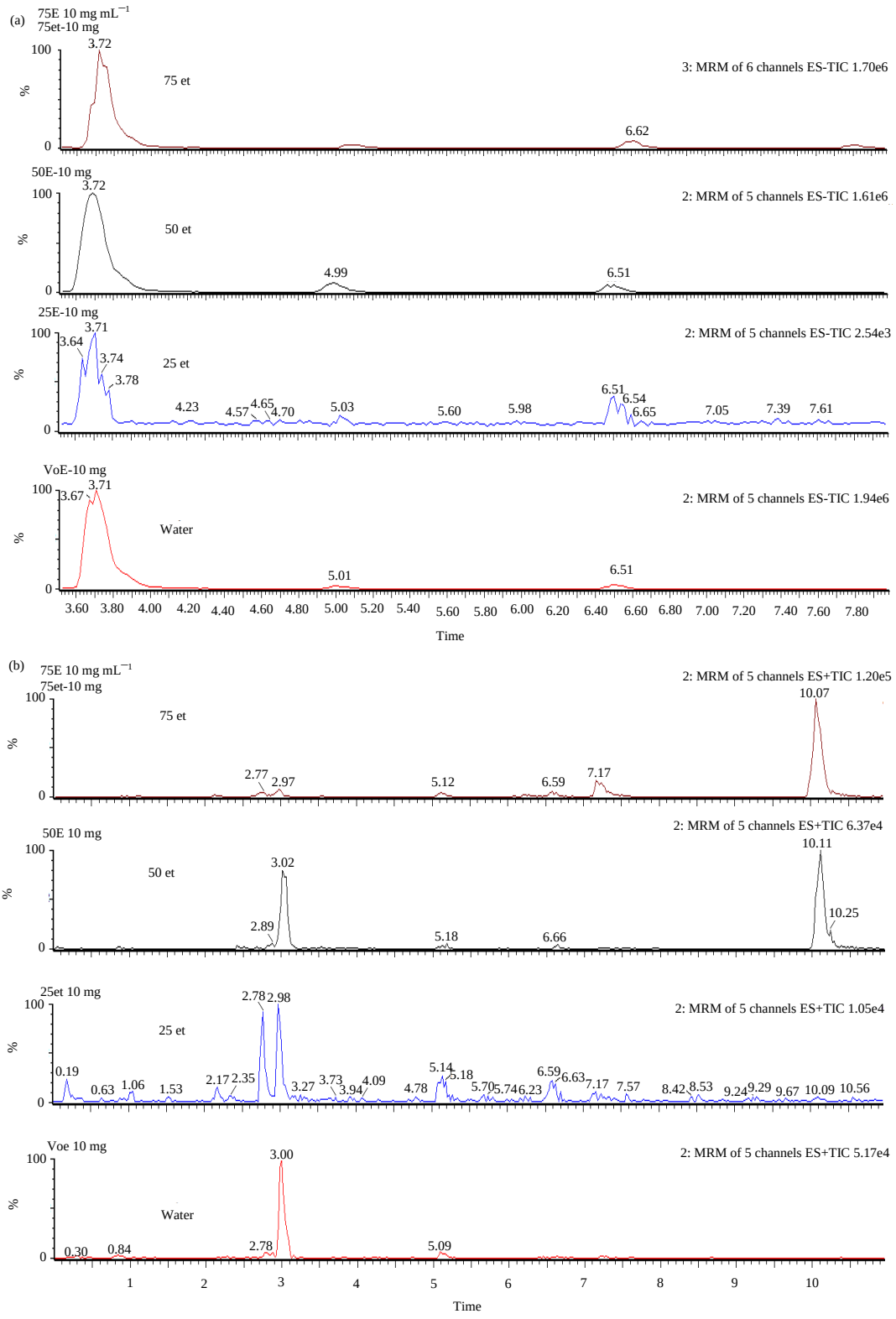
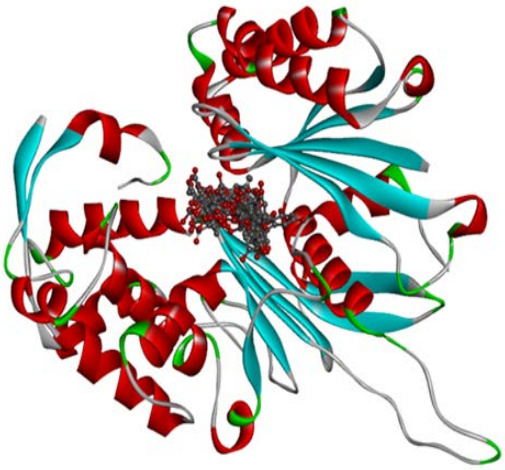
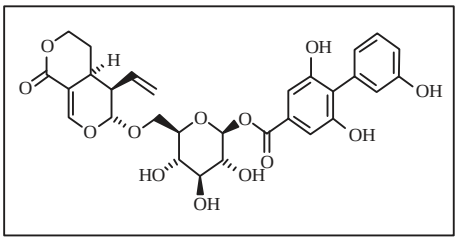


Fig. S2(a-b): TIC chromatograms of water, 25, 50 and 75 ethanol water extracts in (a) ESI negative MRM mode with characteristic precursor and product ions for gentiopicroside (Rt = 3.71), isovitexin (Rt = 5.03), amarogentin (Rt = 6.59) and (b) ESI positive MRM mode with loganic acid (Rt = 3.0) and isogentisin (Rt = 10.11). Mass chromatograms and spectra were recorded and processed using MassLynx NT software (version 4.1)





Mode	Affinity (kcal mol^{-1})	Dist from rmsd l.b.	Best mode rmsd u.b.
1	-9.4	0.000	0.000
2	-9.1	2.323	7.075
3	-9.0	1.999	7.286
4	-9.0	2.188	6.071
5	-8.9	2.362	7.612
6	-8.9	2.506	7.240
7	-8.9	1.933	6.021
8	-8.8	2.188	6.688
9	-8.8	2.089	7.435

Fig. S3: Binding site and binding energies for nine most stable cluster of amarogetin and E-NTPDase2 enzyme

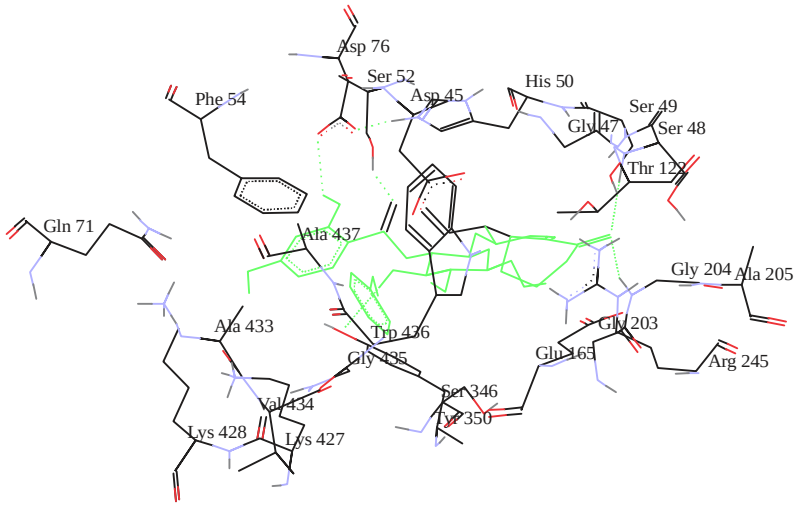
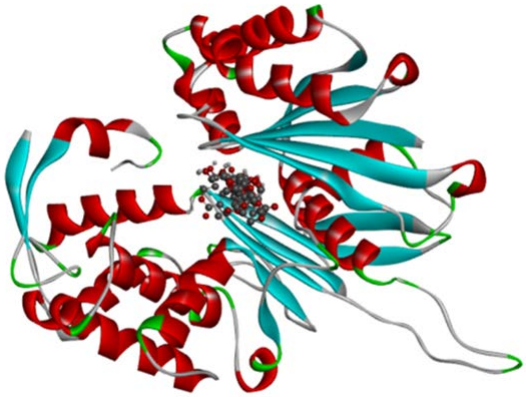
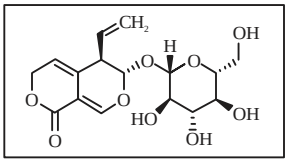


Fig. S4: Structure of amarogentin (displayed by green wireframe style) and amino acid residues in most stable cluster of amarogetin and E-NTPDase2 enzyme (Gln 71, Ala 437, Ala 433, Val 434, Lys 428, Lys 427, Gly 435, Trp 436, Ser 346, Tyr 350, Glu 165, Gly 203, Gly 204, Ala 205, Arg 245, Phe 54, Asp 76, Ser 52, Asp 45, His 50, Gly 47, Ser 49, Ser 48 and Thr 122)





Mode	Affinity (kcal mol^{-1})	Dist from rmsd 1.b.	Best mode rmsd u.b.
1	-8.5	0.000	0.000
2	-8.4	1.760	2.941
3	-8.3	1.583	2.153
4	-8.0	2.604	4.073
5	-7.9	1.869	3.666
6	-7.9	1.950	3.203
7	-7.9	2.099	3.828
8	-7.9	1.691	3.076
9	-7.9	2.097	2.893

Fig. S5: Binding site and binding energies for nine most stable cluster of gentiopicroside and E-NTPDase2 enzyme

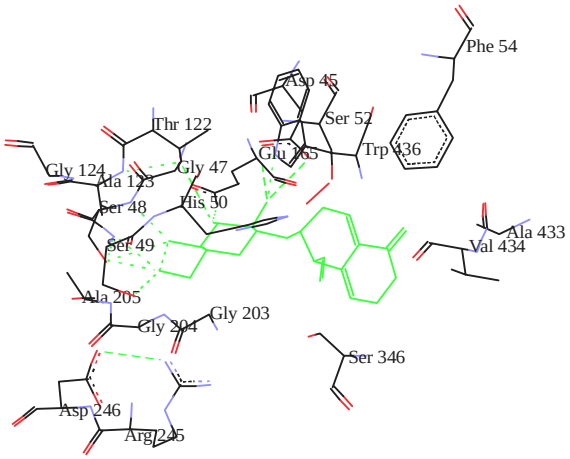
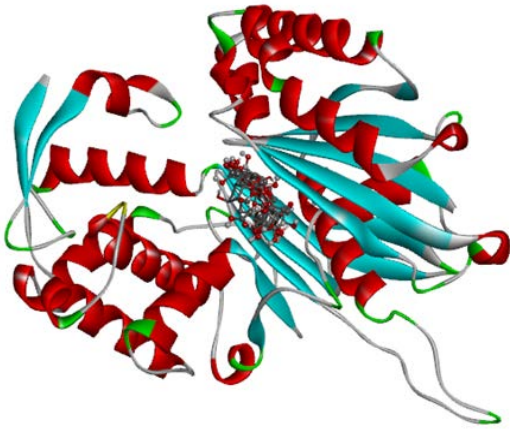
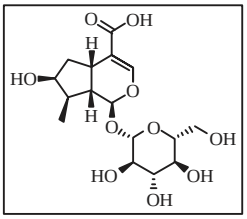


Fig. S6: Structure of gentiopicroside (displayed by green wireframe style) and amino acid residues in most stable cluster of gentiopicroside andE-NTPDase2 enzyme (Gly 204, Gly 203, Ala 205, Asp 246, Arg 245, Ser 346, Val 434, Ala 433, Ser 48, Ser 49, Ala 123, Gly 124, Gly 47, His 50, Thr 122, Asp 45, Glu 165, Ser 52, Trp 436 and Ser 346)





Mode	Affinity (kcal mol^{-1})	Dist from rmsd 1.b.	Best mode rmsd u.b.
1	-8.6	0.000	0.000
2	-8.5	1.867	7.228
3	-8.5	1.757	7.047
4	-8.5	1.611	6.393
5	-8.5	2.009	6.772
6	-8.4	1.875	3.893
7	-8.4	1.952	6.792
8	-8.3	2.080	4.489
9	-8.3	1.322	2.640

Fig. S7: Binding site and binding energies for nine most stable cluster of loganic acid and E-NTPDase2 enzyme

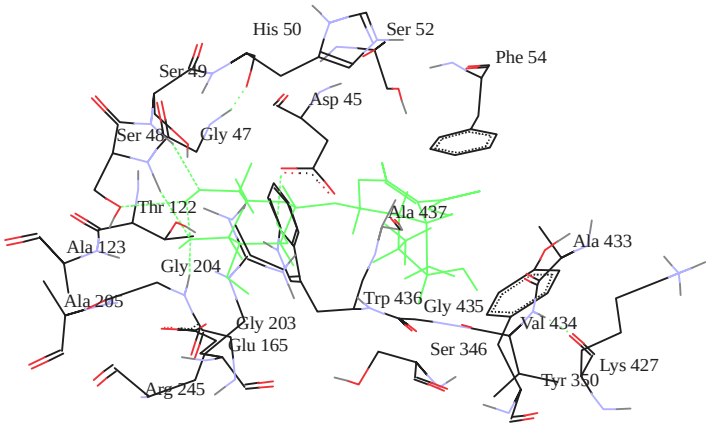


Fig. S8: Structure of loganic acid (displayed by green wireframe style) and amino acid residues in most stable cluster of loganic acid and E-NTPDase2 enzyme (Ala 123, Ala 205, Thr 122, Gly 204, Gly 203, Glu 165, Arg 245, Ala 437, Trp 436, Gly 435, Ser 346, Val 434, Tyr 350, Ala 433, Lys 427, Ser 48, Ser 49, Gly 47, His 50, Asp 45, Ser 52 and Phe 54)

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