

Article

Studies on the Inhibition of Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) by 2-(3,4-Dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one, a Flavonoid from *Pistacia chinensis*

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Abstract: Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) regulates skeletal and soft tissue mineralization by hydrolyzing nucleotide triphosphates and cyclic nucleotides, and is involved in the modulation of immune system. In fact, ENPP1 degrades 2',3'-cyclic GMP-AMP dinucleotide (2',3'-cGAMP), which is an agonist of surface receptor stimulator of interferon genes (STING), thus downregulating immune response. Consequently, ENPP1 inhibitors are being studied as adjuvant agents in infections and cancer. *Pistacia chinensis* is a medicinal plant endowed with several biological activities and traditional uses. In the current study, we report the isolation of transilutin (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one) from the methanolic extract of *P. chinensis* barks and the investigation of its activity as ENPP1 inhibitor. The compound was tested in vitro against snake venom phosphodiesterase, which is structurally related to ENPP1, and dose-dependently inhibited the enzyme. Moreover, molecular modeling studies were employed to assess the binding motif of the transilutin with the macromolecular target. Our findings support the traditional medical application of *P. chinensis* and its extracts by shedding new light on the mechanisms underlying their biological action.

Keywords: ENPP1; phosphodiesterase; *Pistacia chinensis*; transilutin; flavonoids; docking

1. Introduction

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is a protein found in the membrane, bound intracellularly and on the cell surface, and secreted in the extracellular matrix [1]. This enzyme is known for its role in the regulation of skeletal and soft tissue mineralization through the generation of pyrophosphate, an inhibitor of hydroxyapatite formation [2,3]. From the point of view of its molecular mechanism, ENPP1 has a role in purinergic signaling by selectively hydrolyzing its substrates [1]. More specifically, the enzyme has higher affinity for 2',3'-cyclic GMP-AMP dinucleotide (2',3'-cGAMP) than for cAMP [4]. So far, ENPP1 is the only known mammalian hydrolase of 2',3'-cGAMP, while a bacterial enzyme with the same activity but lower efficiency, named SntA, has been reported [5].

In this context, mounting evidence suggests the involvement of ENPP1 in a range of conditions including diabetes, infections, cancer, cardiovascular disease, and osteoarthritis [2]. Indeed, 2',3'-cGAMP stimulates the endoplasmic reticulum surface receptor stimulator of interferon genes (STING) to activate the production of type 1 interferons. Such cytokines trigger an immune system response to virally infected and cancer cells [1]. Thus, by hydrolyzing 2',3'-cGAMP, ENPP1 suppresses this reaction, and cancer aggressiveness has been related to ENPP1 expression [6,7]. Consequently, ENPP1 was recently highlighted as a target for the identification of compounds capable of modulating immune response [1], and this emerging role of ENPP1 pushed the rediscovery of the enzyme in the field of innovative immunotherapies [3].

A very recent example in this context is represented by the work of Chen et al., who reported a novel orally bioavailable small molecule, named OC-1, which is active in preclinical models as an ENPP1 inhibitor for cancer immunotherapy [8]. Indeed, the recent scientific literature is very prolific, and several new research articles concerning this topic were published in 2022 and 2023. Jung et al. reported that compounds based on the scaffold of pyrimidinone can inhibit ENPP1 in vitro and possess antiproliferative properties [9]. With the aid of virtual screening techniques, Wang et al. identified a set of quinazolinone derivatives that stimulate immune response in vivo [10]. Additionally, Jeong et al. described pyrrolopyrimidine and pyrrolopyridine derivatives that are active as ENPP1 inhibitors and that inhibit tumor growth in a mouse model [11], while Gangar et al. designed, synthesized and tested a set of non-nucleotidic thioguanine-based small molecule inhibitors in vivo for application in cancer immunotherapy [12]. In Figure 1, the chemical structures of ARL 67156 and of suramin, examples of nucleotidic and non-nucleotidic ENPP1 inhibitors, are reported.

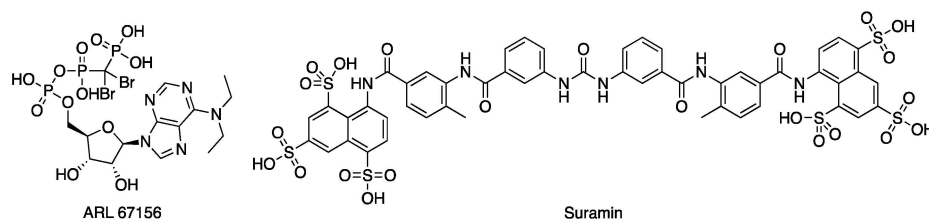


Figure 1. Chemical structure of ARL 67156 and suramin, two compounds investigated as ENPP1 inhibitors [3].

Pistacia chinensis subsp. *integerrima* (J. L. Stewart) Rech. f. belongs to the family of Anacardiaceae and it is native and common in south Asian regions [13,14]. It must be noted that several communities utilize *Pistacia* species for an extensive array of purposes, and thanks to its aesthetically pleasing scarlet or blue fruit and seasonal leaf color change, *P. chinensis* is commonly used in landscaping in temperate regions around the globe. The wood, known for its strength and thickness, is used for various applications. The plant is used as a decorative tree and as a rootstock, and its role as source of biofuels is emerging [15,16]. The aromatic parts such as the leaves, blossoms, and flowers are used for flavoring. Eventually, aromatic oil from fresh leaves has several valuable uses [14].

Most importantly, plants from the genus *Pistacia*, and *P. chinensis* in particular, are known for their folkloric and therapeutic uses in traditional medicine, mainly connected to the immunostimulant, antimicrobial, anticancer, antioxidant, anti-inflammatory, analgesic, antidiarrheal, estrogen-like and muscle relaxant activities of their parts or extracts [14,17,18]. From the point of view of the chemical composition, *P. chinensis* offers a variety of diverse compounds including sterols, terpenoids, and phenolic compounds [19]. In particular, our research group has previously investigated the potential of some flavonoids isolated from this plant as anti-leishmanial agents [20–22].

In the current work, we describe the preparation and fractionation of extracts from the barks of *P. chinensis*. Bark extracts were previously tested for their role in nephroprotection and against lung and thyroid toxicity induced by CCl₄ [23,24]. Additionally, we here report

the isolation of transilitin (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one), a flavonoid which we investigated for its activity against ENPP1. In particular, the compound was tested in vitro for its inhibitory activity against snake venom phosphodiesterase (PDE), and we studied the formation of its complex with human ENPP1 in silico, with the aim of assessing the molecular basis of the protective and immunostimulant properties of *P. chinensis*.

In the past years, transilitin has been investigated as a neuroprotective agent as well as a urease and xanthine oxidase inhibitor, but no reports about its role as a phosphodiesterase inhibitor were retrieved from the literature. On the other hand, 7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, which is another flavonoid that can be extracted from *P. chinensis* galls, showed inhibitory activity towards snake venom PDE in the low μM range [25].

2. Materials and Methods

2.1. Plant Collection, Extraction and Isolation of Transilitin

P. chinensis barks were collected from Hostel 02 University of Peshawar, K.P., Pakistan. The plant specimen was brought to the Department of Botany of the University of Swabi and identified by Dr. Muhammad Ilyas. The voucher specimen number UOS/Bot-55 was deposited in the herbarium of the same department. The extraction procedure was performed in accordance with previously reported protocols, with some modifications to allow the isolation of the desired compound [26]. The barks of *P. chinensis* were washed with water and dried under shade. Some 7.34 kg of the shade-dried plant materials were ground to obtain a fine powder, which was extracted with 100% methanol (15 L) at room temperature for 16 days. The obtained extract was filtered and concentrated under vacuum, and the resulting crude extract (81.29 g) was then suspended in water (200 mL) in a separating funnel and partitioned with equal amounts of *n*-hexane, chloroform, and ethyl acetate, which were used in sequence to obtain different fractions. Thin layer chromatography (TLC, chloroform/methanol 90:10) was used to assess the presence of the compound in the solutions in comparison with the standard compound. Consequently, the ethyl acetate fraction was evaporated to provide the corresponding extract (6.43 g) upon evaporation of the solvent. Thus, this crude product was subjected to column chromatography over silica gel. The column was eluted with chloroform/methanol (96:4), and a fraction rich in the desired compound was isolated. Recrystallization from chloroform/methanol (1:1) provided pure transilitin (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one; CAS number 26788-86-3, PubChem CID 44258702. The identity of the compound was assessed via mass spectrometry and NMR analysis, which were in agreement with a previous report in which the compound is described as 7,8,3',4'-tetrahydroxy-3-methoxyflavone [27].

2.2. In Vitro Inhibition of PDE I

In this assay, the activity of the extracts and of the isolated compound transilitin against PDE I from *Crotalus adamanteus* (EC 3.1.4.1) was assayed by following a standard protocol [28–30]. The enzyme with the product code P3134 was obtained from Merck (Darmstadt, Germany). TRIS buffer, magnesium acetate, ammonium acetate and *bis*(*p*-nitrophenyl)phosphate were purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Briefly, 33 mM Tris-HCl was used as a buffer (pH = 8.8) and 30 mM magnesium acetate was added as a cofactor, with snake venom PDE at 0.000742 U as the final amount. Then, 0.33 mM *bis*(*p*-nitrophenyl)phosphate was employed as a substrate, while disodium EDTA was used as the positive control. The test compounds, diluted in buffer from DMSO stocks (for transilitin and EDTA, 0.00625–0.2 mg/mL, see Figure S1 in the Supplementary Materials), were incubated in a 96-well plate with the enzyme. The formation of the product of the reaction catalyzed by the enzyme was monitored with the addition of the substrate at 1 min intervals, after an incubation period of 30 min at 37 °C. A

Spectramax 384 reader (Molecular Devices, San Jose, CA, USA) was used for the detection at 410 nm. The % inhibition was calculated by using the following equation:

$$\% \text{ Inhibition} = 100 - (\text{Absorbance of sample} / \text{Absorbance of control} \times 100)$$

All the reactions were performed in triplicate. IC₅₀ values were calculated using the Enzyme Kinetics BestCurvFit software (Version 9.2, EZ-Fit) [31].

2.3. Computational Studies

The 3D X-ray crystal structures of human ENPP1 bound to adenosine-5'-thio-monophosphate were retrieved from the RCSB Protein Data Bank (www.rcsb.org; accessed on 25 July 2023; PDB ID 6WEU, resolution 2.65 Å) [1]. Before site-specific docking studies, the protein was processed through the DockPrep tool of UCSF Chimera to fix potentially missing residues in the structure [32], and docking was performed using AutoDock Vina [33]. The docking grid was centered on the cognate ligand binding site and a receptor search volume space was created with the following coordinates and dimensions: x = 17.8363, y = −34.8195, z = −29.8274; size: 20.0000 × 20.0000 × 20.0000 Å. The number of generated docking poses was set to 10, and the docking energy conformation value was expressed in kcal/mol. The best scoring pose was selected for further analysis. Residue numbering used in the PDB file was adopted. To validate the adopted site-specific molecular docking protocol, the cognate ligand was redocked, and the root-mean-square deviation (RMSD) value was calculated. A RMSD value of 1.704 was retrieved for the best scoring pose, thus confirming the reliability of the search algorithm. Results and graphic visualizations were processed using UCSF Chimera [32].

MD simulations were carried out using PlayMolecule (Acellera, Stanmore, UK), starting from the output models of docking experiments. The ligand was prepared by running a Parametrize function based on a GAFF2 force field [34]. The complex was then optimized for the simulation using ProteinPrepare and SystemBuilder functions, setting a cubic solvent box, pH = 7.4, neutralization with 0.15 M NaCl, an AMBER 14SB force field, and default experiment parameters [35]. Simulations (2 ns) were carried out using SimpleRun, with default settings [36]. Plotting of the root mean square deviation (RMSD) was performed using Excel 15.31 (Microsoft, Redmond, WA, USA).

Physicochemical descriptors relevant for drug-likeness, ADME parameters, and pharmacokinetic properties were computed using the SwissADME web service (www.swissadme.ch, accessed on 19 September 2023. Molecular Modeling Group—Swiss Institute of Bioinformatics, Lausanne, CH) [37]. Ligand-based target prediction studies were performed by using the SwissTargetPrediction web service (www.swisstargetprediction.ch, accessed on 19 September 2023. Molecular Modeling Group—Swiss Institute of Bioinformatics, Lausanne, CH) [38]. In these experiments, a canonical SMILES string for transilitin (COC1=C(OC2=C(C1=O)C=CC(=C2O)O)C3=CC(=C(C=C3)O)O) was used as input for the calculations.

3. Results and Discussion

3.1. Extraction of Transilitin

P. chinensis is a well-known medicinal plant used for the treatment or management of various conditions such as diabetes, diarrhea, inflammation, asthma, fever, pain, and liver diseases. Several chemically diverse bioactive compounds with a plethora of potential pharmacological applications have previously been extracted from this natural source [14]. In the current study, transilitin (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one, Figure 2) was isolated from the methanolic fraction of the extract of *P. chinensis* barks. The purification of the compound required subsequent steps, which included fractionation, column chromatography over silica gel, and eventually, recrystallization. The identity and purity profile of the extracted compound were assessed via physical and spectroscopic analysis, which were in agreement with previously reported data [26,39].

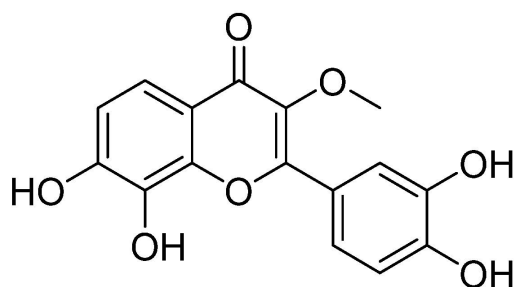


Figure 2. Chemical structure of compound transilitin (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one) isolated from *P. chinensis*.

Transilitin is a natural flavone also found in other plants, and it is known for its several biological properties. It has been previously mostly investigated as a neuroprotective agent, inhibiting amyloid beta aggregation in Alzheimer's disease and contrasting the formation of amyloid fibrils in Parkinson's disease models [40–42]. The compound was also reported to inhibit urease [26] and xanthine oxidase [43,44].

3.2. In Vitro Inhibition of PDE

The identification of nonhydrolyzable ENPP1 inhibitors is a strategy pursued in medicinal chemistry to stimulate innate immune response, a biological event which could be a valuable support in cancer treatment and against infections [45]. Currently, nucleotide-based and non-nucleotide-based ENPP1 inhibitors are being studied [3]. In this context, the aim of this study was to assess the inhibitory activity of transilitin isolated from *P. chinensis* barks towards snake venom PDE, in agreement with previous studies from some of our research group that demonstrate the role of flavonoids from this plant as PDE inhibitors [25]. As shown in Figure S1 in the Supplementary Materials, the tested compound inhibited the enzyme dose-dependently. Disodium EDTA was used as a positive control in this assay. In fact, by acting as a metal chelator, this compound is known to inactivate PDEs [46]. The results of this assay are reported in Table 1. On the other hand, *n*-hexane, chloroform, ethyl acetate, and methanol extracts showed a maximum inhibitory effect of 39.09%, 70.23%, 79.12%, and 85.66% on the enzyme when tested separately (0.2 µg/mL of extract in buffer).

Table 1. Inhibition of snake venom PDE activity by transilitin isolated from *P. chinensis*. Disodium EDTA was used as a positive control in this study.

Compound	IC ₅₀ (µM)
Transilitin	284.0 ± 2.0
Disodium EDTA	263.3 ± 2.2

3.3. Computational Studies

Given the promising in vitro results, a molecular modeling study was performed to provide insights into the interaction of transilitin with the target protein. More specifically, the compound was docked to the active site of human ENPP1, and the binding mode was analyzed.

The site-specific docking study was performed using AutoDock Vina [33], and the protocol was validated using re-docking adenosine-5'-thio-monophosphate. This compound represents the cognate ligand, which is the co-crystallized molecule present in complex with the protein in the PDB file (PDB ID 6WEU) [1]. The similarity between snake venom PDE and human ENPP1 was assessed via BLAST alignment (www.uniprot.org/blast/, accessed on 25 September 2023) showing a 52.4% sequence identity between J3SEZ3 PDE1_CROAD venom phosphodiesterase 1 from *Crotalus adamanteus* (Eastern diamondback rattlesnake) and P22413 ENPP1_HUMAN Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 from *Homo sapiens*. Additionally, we assessed 3D structure similarity using the

UCSF Chimera MatchMaker function by comparing the human 6WEU model with the one computed by AlphaFold (alphafold.ebi.ac.uk/entry/J3SEZ3, accessed on 14 September 2023) for snake venom PDE I, and a root-mean-square deviation (RMSD) value of 0.730 was computed (Figure S2 in the Supplementary Materials). It must be also noted that besides ENPP1, snake venom PDE is also structurally related to ENPP3, as noted by Pan et al. [47].

The results of the docking study are depicted in Figure 3.

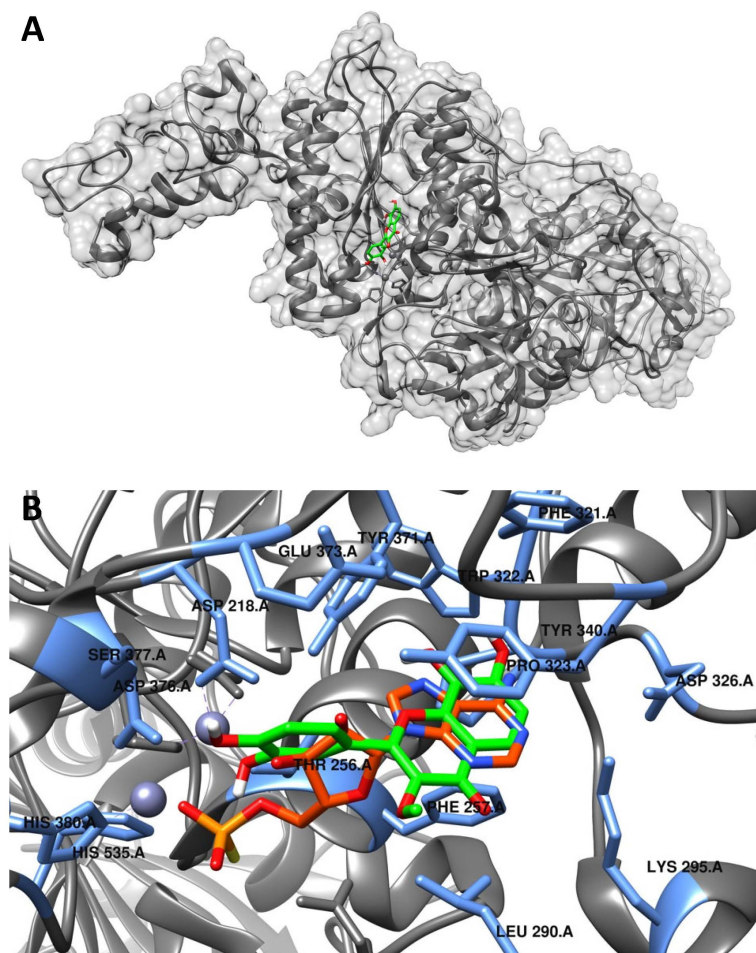


Figure 3. Predicted interaction motif of transilitin (green) with ENPP1 (A). A detailed view of the binding mode of the studied compound in comparison with the cognate ligand (orange) is also presented. The interacting residues have been highlighted and labeled (B).

A calculated binding energy value of -8.3 kcal/mol was computed for transilitin, which overcomes that predicted for the re-docked cognate ligand (-7.9 kcal/mol). Notably, the two compounds share a common binding motif. In particular, the bicyclic scaffolds of the two compounds are superimposed and interact with the aromatic residues Phe257 and Tyr340. On the other hand, the catechol ring of transilitin points towards the Zn^{2+} ions, somehow mimicking the positioning of phosphate groups of the substrate, thus suggesting a potential activity in interfering with enzymatic activity. It should be also pointed out that in the case of competitive inhibition, as suggested by the substrate-like binding predicted by docking experiments, the IC_{50} value would be strictly dependent on the concentration of substrate used in the inhibition analysis.

As docking studies indicated that transilitin could efficiently interact with ENPP1, a preliminary short molecular dynamics (MD) simulation was set up in order to investigate the stability of the computed ligand–target complex. The experiment showed that the complex formed by the natural compound and the macromolecular target promptly points toward stabilization (Figure S3 in the Supplementary Materials). In any case, must be noted

that even if very limited RMSD fluctuations can be observed for both alpha carbons and the sidechains of the macromolecule, the ligand did not reach full stabilization in this limited timeframe (Figure S3A). Indeed, the analysis of the main clusters of the MD trajectory demonstrated that a rearrangement of the molecule occurs, even if no rotation or expulsion from the binding site could be observed (Figure S3B).

Additionally, given the promising in vitro results and structure-based computational data, ligand-based drug discovery tools were employed to evaluate the potential of transilitin as a molecule with adequate features for therapeutic applications.

First, physicochemical descriptors relevant for ADME parameters and pharmacokinetic properties were predicted using the SwissADME web service [37]. The considered features allow preliminary evaluation in silico if a molecule possesses the proper chemical features to encourage its development as a drug to be potentially administered orally. The ideal values for such physicochemical descriptors are detailed in Lipinski's rule, which states that the considered compound should possess no more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, a molecular weight below 500 Da, and an octanol/water partition coefficient (LogP) that does not exceed 5 [48]. In general, the definition of "drug-likeness" relates to the similarity of a drug and its properties to most existing drugs; if the compound follows Lipinski's rule, its oral bioavailability can be hypothesized. In the case of transilitin, the compound has a molecular weight of 316.26 Da, four hydrogen bond donors, and seven acceptors. Moreover, a LogP value of 1.52 was calculated. Thus, the molecule possesses the ideal chemical features to be defined as a "drug-like" compound. Additionally, it must be noted that the compound is classified as water-soluble, and water solubility is a crucial aspect for bioactive molecules. Notably, transilitin was also predicted to be absorbed through the gastrointestinal tract, while no passive permeation through the blood–brain barrier was highlighted by the software.

Then, we used the SwissTargetPrediction web service to investigate other potential macromolecular interactors for transilitin. This software works by using the "similarity principle", assuming that similar compounds will share similar protein targets [38]. Interestingly, beta amyloid A4 protein, which is considered a hallmark protein for Alzheimer's disease in the human brain [49], was highlighted as the target with the highest probability. It must be noted that anti-aggregatory and neuroprotective properties have been previously reported for flavonoids. Interestingly, transilitin was tested along with quercetin and other analogues in vitro, and the studied compound significantly inhibited beta amyloid aggregation and counteracted toxicity in PC12 cells [42]. Similarly, Churches et al. reported the anti-aggregatory potential of transilitin by means of computational and experimental investigation [40]. Additionally, aldose reductase was predicted as a target of medium probability, while other proteins such as NADPH oxidase 4 showed lower probability. Again, it should be considered that flavonoids have been previously reported as ligands and inhibitors of aldose reductase, and their contribution to the development of compounds against diabetic complications has been proposed [50,51]. Even if it is very preliminary, this target prediction study further supports the potential of transilitin and agrees with literature data, as this compound may synergically act on targets of relevance in pathogenic conditions.

4. Conclusions

Our research revealed that the methanolic extract of *P. chinensis* bark contains a flavone, named transilitin, which possesses a notable inhibitory role against ENPP1, an enzyme involved in the regulation of immune response. Still, the inhibitory activity was observed to be in the μM range in vitro against snake venom PDE, and thus it should be improved in semi-synthetic derivatives before future pharmacological applications.

Computational studies, based on docking and preliminary MD simulations, showed that the compound efficiently interacts with the residues located in the active site of the target protein, partially sharing the binding motif of the cognate ligand, thus supporting the potential of this molecule and of its structural features. Moreover, ligand-based com-

putational studies have highlighted that transilitin has the proper chemical features to be defined as a “drug-like” compound, and that the compound shows a high probability of interacting with beta amyloid A4 protein, a target of primary relevance in Alzheimer’s disease.

These findings pave the way for the development of novel ENPP1 inhibitors based on the flavonoid scaffold, and additionally, the current study rationally supports the traditional protective and immunostimulant roles of *P. chinensis*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemistry5040142/s1>, Figure S1: Inhibition of PDE by EDTA and transilitin in vitro, Figure S2: Structural comparison of 3D protein models, Figure S3: Results of MD simulation.

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