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Oleuropein and Verbascoside - Their Inhibition Effects on Carbonic Anhydrase and Molecular Docking Studies

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Abstract: Recently, carbonic anhydrase (CA, E.C.4.2.1.1) inhibitors from natural product have paved the way for novel drug design in the treatment and prevention of some global diseases such as glaucoma, diabetes, and cancer. For this purpose, the inhibition effects of oleuropein and verbascoside from olive (*Olea europaea* L.) oil on human carbonic anhydrase I, and II (hCA I, and II) isoenzymes were evaluated in the current study. The inhibition effects of both natural compounds were determined by the esterase activity (*in vitro*). IC_{50} value of oleuropein and verbascoside was calculated as 1.57 and 1.73 µM for hCA I isoenzyme, respectively. At the same manner, K_i values were determined as 1.25 ± 0.42 and 2.00 ± 0.42 µM, respectively. Then, IC_{50} value of each compound for hCA II isoenzyme was calculated as 2.23 and 1.90 µM, respectively. Similarly, K_i values were determined as 2.37 ± 0.87 µM and 1.49 ± 0.33 µM, respectively. Also, the inhibitory effects and potent binding mechanisms of oleuropein and verbascoside on hCA I, and II isoenzymes were realized by molecular docking studies. Consequently, both natural phenolic compounds demonstrated the potent inhibition profiles against the both isoenzymes. Therefore, we believe that these results may break new ground in the drug development for the treatment of some global disorders.

Key words: oleuropein, verbascoside, carbonic anhydrase, enzyme inhibition, molecular docking

1 Introduction

Enzymes are responsible for all chemical reactions necessary to sustain life, and regulate many activities, which are liable to carrying out biochemical process[1)]. Thus, enzyme inhibition studies have practical significance in some global diseases such as glaucoma, diabetes, and cancer[2, 3)]. Most of diseases may be associated with either excessive activity of an enzyme or a relative and/or absolute deficiency of one or more enzymes. In addition, many drugs exert their biological effects through interactions with enzymes.

The hCAs are a large family of Zn^{2+} ion-containing metalloenzymes that involved in the catalysis of the reversible interconversion of carbon dioxide into bicarbonate and proton[3, 4)].

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

To date, sixteen CA isoenzymes found in humans belong to α-CA family, which differ in tissue distribution and subcellular localization[5, 6)]. These isoenzymes provide carbon dioxide and ion transport, respiration, fluid balance and acid-base balance in many tissues[3)]. They are also involved in biosynthetic reactions such as gluconeogenesis, lipogenesis, and urea synthesis as well as in many pathological and physiological events such as tumor formation and calcifica-

Abbreviations: CA: Carbonic anhydrase; hCAs: Human carbonic anhydrases; CA I: Carbonic anhydrase inhibitor; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; RBCs: Red blood cells; IC_{50}: Half-maximal inhibitory concentration; K_i: Inhibition constant; ADT: Auto dock tools; PMV: Python Molecule Viewer

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tion[4]. CAs are significant for the treatment and prevention of some diseases such as osteoporosis, epilepsy, cancer, glaucoma, edema, obesity, and Alzheimer's disease. Therefore, inhibition or activation of these isoenzymes can clinically be significant[3]. CA inhibitors (CAIs) are potential therapeutic targets in the treatment of many diseases. However, their use can cause adverse effects in some cases[7, 8]. Sulfonamides have been in clinical use as CAIs for about 75 years, but they are often associated with a variety of side effects, among others, they contain hypersensitivity[9–11]. In addition, the use of CAIs may increase blood glucose concentrations in patients with diabetes, increase the risk of electrolyte imbalance in patients with liver failure, promote the development of calcium oxalate kidney stones in patients with kidney disease or stones, and make the condition worse. In addition, some side effects may occur that usually do not need medical attention. For instance, the clinically used CAIs may cause numbness in mouth and lips, tingling in fingers and hands, nausea, vomiting, loss of appetite, loss of taste and smell, fatigue, headache, blurred vision, tinnitus, and diarrhea[12]. They can also cause allergic reactions such as anaphylaxis and rash, as well as serious effects such as hypokalemia, metabolic acidosis, the deficiency of all blood cells, and more[13, 14]. Therefore, in recent years, scientists have been looking for new and more effective CAIs, and fundamental research has focused on using natural products for CAIs. Recently, the inhibition effects of many compound on hCA I, and II isoenzymes have been reported[11, 15–18]. The focus of the current study is on the potential inhibition effects of oleuropein and verbascoside against hCA I, and II isoenzymes, which have crucial effects in medicinal aspects.

Ever since ancient times, for different purposes like human health and well-being, olive (*Olea europaea* L.) oil has been used. The olive oil has long been accepted as one of the herbal products widely used in traditional medicine[19]. It contain many bioactive compounds such as oleuropein, verbascoside, tyrosol, hydroxytyrosol, diosmetin, luteolin, and rutin. Among them, oleuropein possesses beneficial effects on human health and has various biological properties that comprise antioxidant[20], antidiabetic[21, 22], anticancer[23], cardioprotective[24], neuroprotective[24], and hepatoprotective[25] effects. These properties are probably due to the chemical structure of oleuropein, which include hydroxytyrosol and elenolic acid[26]. On the other hand, verbascoside that known as acteoside is another phenolic glycoside present in olive leaves. It is a phenylethanoid consisting of a cinnamic acid and hydroxyphenylethyl moieties attached to a β-glucopyranose through a glycosidic bond[27, 28]. This compound possesses various biological activity including antioxidant[29, 30], antimicrobial[31], anti-inflammatory[32] and antimutagenic[33] properties.

As we know, this research is the first study dealing with the inhibition effects of oleuropein and verbascoside as main natural phenolic compounds in olive oil against hCA I, and II isoenzymes. In order to detail inhibitory effects of oleuropein and verbascoside on the binding sites of both isoenzymes, molecular docking studies were also carried out.

2 Materials and Methods

2.1 Extraction and purification of CA isoenzymes

The purification procedure was carried out according to the method described in previous studies[16]. The procedure for the preparation of CA hemolysate from red blood cells (RBCs) was carried out according to the method described in literature[16, 34]. In order to separate RBCs from plasma, the blood samples were centrifuged at 2500 × g for 15 min at +4℃, and washed three times with an aqueous solution of 0.9 percent sodium chloride. The washed-samples were lysed in three volumes of ice-cold water for 45 min. To remove cellular remnants, the hemolysate was centrifuged at 20000 × g for 30 min at 4℃. After that, the purification steps were deployed to elute both CA isoenzymes. The hemolysate was adjusted to pH 8.7 with solid Tris, and applied to Sepharose-4B-L-Tyrosine-sulfanilamide affinity column for selective retention of both hCA isoenzymes. The hCA I, and II isoenzymes were fractionated with different buffers throughout the column, and each isoenzyme was eluted, separately. The eluents were pooled in different test tubes and their absorbance was recorded at 280 nm as the reference wavelength[16]. Following the purification steps, for checking of purities of both isoenzymes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and a single band was observed for each CA isoenzyme (data not shown). This method was carried out according to Laemmle's procedure described in literature[17, 35, 36].

2.2 CA inhibition assay

The isoenzyme esterase activities were determined according to the method, which described in the literature[16, 34, 37]. Based on this method, *p*-nitrophenylacetate was used as a substrate and transformed to *p*-nitrophenolate ion as a function of CA isoenzymes. The absorbance values of *p*-nitrophenolate were spectrophotometrically measured at 348 nm in a kinetic mode for 3 min (at 25℃)[16, 36]. Acetazolamide was used as a standard inhibitor for hCA I, and II isoenzymes. In the presence of oleuropein and verbascoside, the activities of hCA I, and II isoenzymes were determined. An activity (%)-[Oleuropein or verbascoside] graphs were drawn to calculate the inhibition effects of both compounds on hCA I, and II isoenzymes. The IC_{50} values, the concentration required to produce half-maximum inhibition, were obtained from percent inhibition graph for each compound. K_i values, the indication of the

potency of an inhibitor, have been obtained from Lineweaver-Burk curves. The inhibition types were found from this graph[17, 38]. In the current study, *p*-nitrophenylacetate was used as a substrate at five different concentrations. Each compound was tested in triplicate at each used concentration, and three different concentrations were used to calculate K_i values. Also, total protein contents in the purification steps were spectrophotometrically determined at 595 nm according to the Bradford method[36, 39].

2.3 Docking studies

Molecular docking was performed to identify binding affinity and possible interactions between the compounds (Oleuropein and Verbascoside) and the isoenzymes (hCA I, and II) by using AutoDockTools (ADT ver.1.5.6). At the molecular docking studies, the interaction of each compound with the active site of each isoenzyme was separately carried out. The x-ray crystal structures of hCA I, and II isoenzymes were obtained from RCSB Protein Data Bank (PDB code: 4WR7 and 3HS4, respectively). These structures in PDB format were passed to ADT. After removing water molecules, and adding only polar hydrogen atoms and Kollman charges to the isoenzyme structures, the structures were saved as PDBQT, an extended PDB format used for coordinate files, which includes atom types and atomic partial charges. The chemical structure of acetazolamide was taken from the DrugBank (https://www.drugbank.ca/), and the structures of oleuropein and verbascoside from PubChem (https://pubchem.ncbi.nlm.nih.gov/). These structures were shown in Fig. 1. After evaluating torsions of the compounds, the compounds were saved as PDBQT file by using ADT. To definite the active site of each isoenzyme, their structures were embedded in a three-dimensional grid, which is $80 \times 80 \times 80$ points at the center via a grid box, which launches interactive commands for setting the grid dimensions and center. Docking studies were carried out using the most efficient method, Lamarckian genetic algorithm with local search, with a total of 10 runs. During the docking process, ten conformers were considered for the compounds. Finally, the conformer with the lowest binding free energy was evaluated by using Python Molecule Viewer (ver.1.5.6) and PyMOL (ver. 2.3.3, Schrödinger, LLC).

2.4 Chemicals

Sepharose-4B, Sephadex G-150, *p*-nitrophenylacetate, chemicals for electrophoresis, oleuropein and verbascoside were purchased from Sigma Chem. Co., USA. All other chemicals were purchased from Merck (Kenilworth, NJ). Blood samples were supplied from the Blood Center of Education and Research Hospital at Ataturk University.

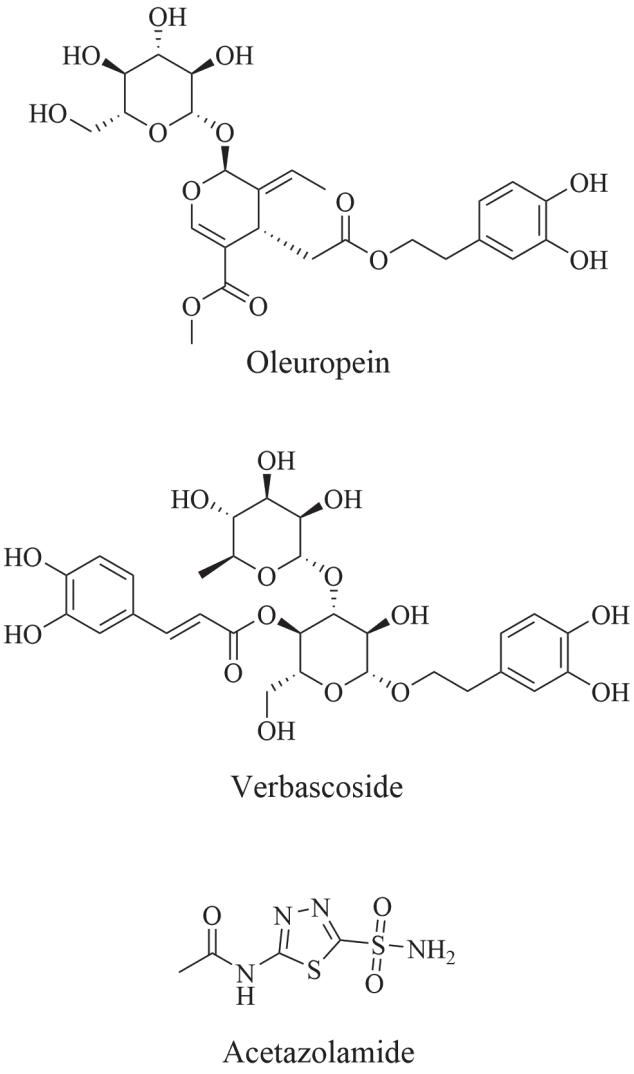

Fig. 1 Chemical structures of oleuropein, verbascoside and acetazolamide.

3 Results and Discussion

3.1 Inhibition studies

CA is a good model for inhibition studies due to its defined structure and active site in detail, the well-known mechanism of its catalytic activity, and many such reasons. Moreover, it is a single-chain protein, which has no disulfide bonds, and can be inhibited or induced by drugs[40]. CAIs tightly bind to the Zn^{2+} ion within the enzymatic active site, and block their activity. Its inhibition is essential for the treatment of some diseases, including epilepsy and glaucoma[3, 40]. Although this enzyme has sixteen isoenzymes, two of them can be easily purified under our laboratory conditions, with the two most significant isoenzymes being hCA I, and hCA II. These isoenzymes have been as well-characterized structurally, and are generally used together in inhibition studies[40]. Therefore, the focus of the current study is on the *in vitro* inhibitory effects of oleuropein and verbascoside on both hCA I, and II isoenzymes.

In recent literature, there are many studies, which have conducted the significant inhibitory properties of phenolic compounds, the potential to become a new class of CA inhibitor[41]. Oleuropein and verbascoside as phenolic compounds have similar structures with *ortho*-diphenolic group and a glycoside residue. According to our results,

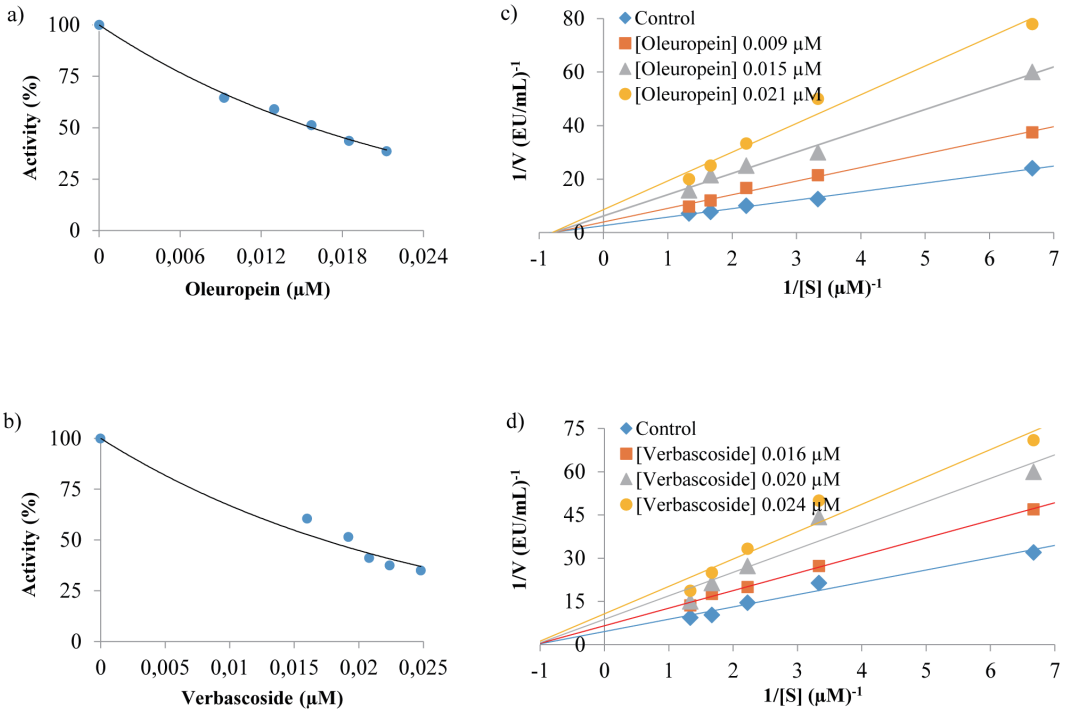

Fig. 2 (a-b) Activity (%)-[Natural compound] regression analysis graphs for hCA I. (c-d) Lineweaver-Burk graphs of oleuropein and verbascoside for hCA I.

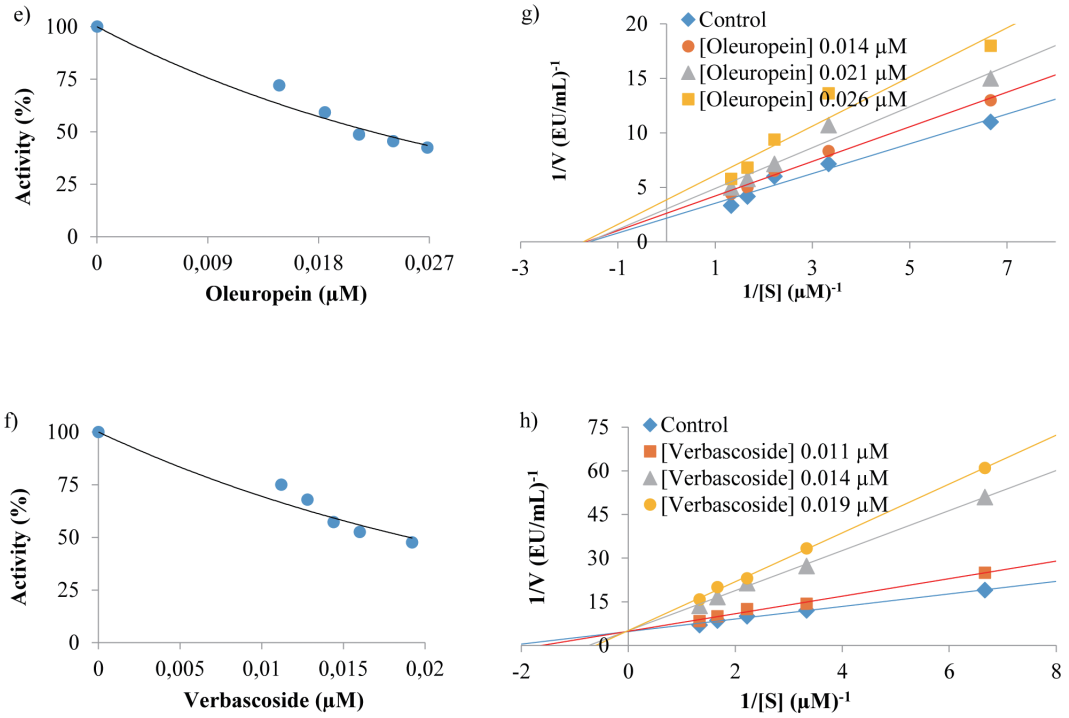

Fig. 3 (e-f) Activity (%)-[Natural compound] regression analysis graphs for hCA II. (g-h) Lineweaver-Burk graphs of oleuropein and verbascoside for hCA II.

oleuropein and verbascoside exhibited strong inhibitory effects against hCA I (Fig. 2), and II isoenzymes (Fig. 3). In the current study, for each compound, IC_{50} and K_i values were calculated, and the type of inhibition was determined. IC_{50} values of hCA I isoenzyme for oleuropein and verbascoside were determined as 1.57 and 1.73 μM, respectively. Oleuropein and verbascoside had the strong inhibition potency against hCA I with K_i values of 1.25 ± 0.42 and 2.00 ± 0.45 μM, respectively. Moreover, oleuropein seemed to be more effective than verbascoside, inhibiting hCA I isoenzyme in the micromolar range. Oleuropein demonstrated about 1.60 times a better hCA I inhibition profile when compared to verbascoside. The strong inhibition effect of oleuropein on hCA I is probably due to carboxylate, methylene, and phenyl groups present in its structure. Additionally, IC_{50} values of hCA II isoenzyme for oleuropein and verbascoside were calculated as 2.23 and 1.90 μM, respectively. At the same manner, K_i value was determined as 2.37 ± 0.87 and 1.49 ± 0.33 μM, respectively. According to these results, verbascoside was found more effective than oleuropein against hCA II isoenzyme, and showed about 1.59 times a better inhibition effect than that of oleuropein. This may be due to the position of carboxylate group and phenyl moiety in the structure of verbascoside. Oleuropein is a phenolic secoiridoid glycoside that consists of a polyphenol, namely hydroxytyrosol, elenolic acid and a glucose molecule. Verbascoside is a glycoside and consists of a heterosidic ester of caffeic acid and hydroxytyrosol. Although oleuropein and verbascoside have similar phenolic moieties[42], these two compounds had slightly advantages over each other, and significant differences for the inhibition of hCA I, and II isoenzymes. In addition, their inhibitory effects were weak compared to that of acetazolamide which is still in clinical used as a specific inhibitor of CA isoenzymes[40]. The inhibition type of oleuropein was found as noncompetitive for both isoenzymes. Verbascoside showed noncompetitive inhibition for cytosolic hCA I isoenzyme, but its inhibition type was found as competitive inhibition against dominant cytosolic hCA II isoform. All inhibition data were summarized in Table 1.

3.2 Molecular docking studies

The identify of active sites of both cytosolic hCA I, and II isoenzymes was done with the reference drug, acetazolamide, according to the docking procedures mentioned in the M&M. Then, oleuropein and verbascoside were docked into the defined active sites, and the binding free energy scores were calculated by using ADT. According to our results, there was a strong agreement between the binding free energy scores (Table 2) and the experimental inhibition results (Table 1). Oleuropein had lower energy score (−7.77 kcal/mol) than verbascoside (−6.18 kcal/mol) against hCA I isoenzyme. Thus, this confirmed the strong interactions of oleuropein with the active site of the 4WR7. The phenyl moiety of oleuropein realized a π-stacking interaction with the side chain of the amino acid His94 (Fig. 4). According to Fig. 4, the carboxylate group of oleuropein realized a salt bridge with the imidazole ring of the amino acid His200. On the other hand, oleuropein had three hydrophobic interactions that were realized by the methylene group and phenyl ring of the compound with the amino acids Val143, Leu198, and Trp209, as shown in Fig. 4. In addition, the hydroxyl groups of oleuropein realized seven hydrogen bondings with the nitrogen atoms of the amino acids Trp5, His64, Thr199, and His200.

According to verbascoside docking result, the ose moiety of verbascoside realized one π-stacking interaction with the phenyl moiety of the amino acid Phe91 (Fig. 5). Besides, verbascoside had seven hydrophobic interactions with the side chains of the amino acids Phe91, Ala121, Ala135, Val143, Leu198, Pro202, and Tyr204. On the other hand, the hydroxyl groups of verbascoside exhibited seven hydrogen bondings with the oxygen atoms of the amino acids His67, Asn69, Gln92, Ser197, and Thr199.

Table 2 Binding free energy scores (S) in kcal/mol of oleuropein and verbascoside in carbonic anhydrase (hCA I, and II) binding sites.

Compounds	Energy score (S) (kcal/mol)	
	hCA I	hCA II
Oleuropein	−7.77	−8.58
Verbascoside	−6.18	−10.08

Table 1 Inhibition effects of oleuropein, verbascoside and acetazolamide on hCA I, and II isoenzymes.

Compounds	IC_{50} (μM)				K_i (μM)	
	hCA I	r^2	hCA II	r^2	hCA I	hCA II
Oleuropein	1.57	0.9933	2.23	0.9518	1.25 ± 0.42	2.37 ± 0.87
Verbascoside	1.73	0.9433	1.90	0.9248	2.00 ± 0.45	1.49 ± 0.33
Acetazolamide[a)]	1.008	0.9935	0.222	0.9943	0.734 ± 0.12	0.159 ± 0.04

[a)] Acetazolamide was used as a standard inhibitor for both hCA I, and II, and taken from reference of the previous study[43].

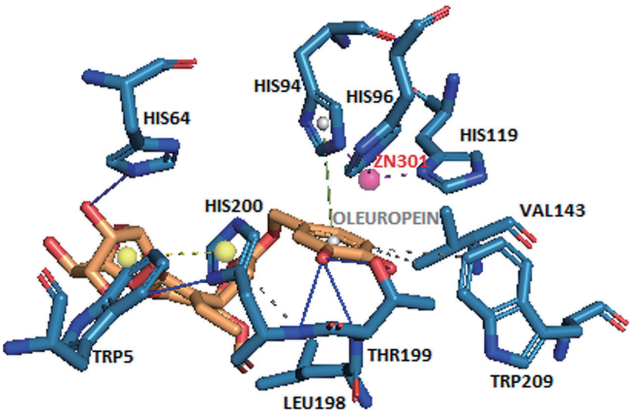

Fig. 4 Schematic presentation of interactions between oleuropein and hCA I(4WR7). Blue color represents hydrogen bondings, grey color represents hydrophobic interactions, green represents π-stacking interactions, and yellow represents salt bridges.

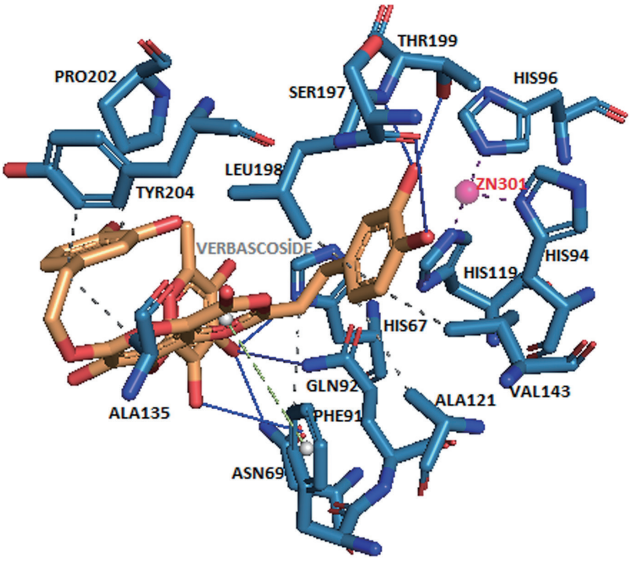

Fig. 5 Schematic presentation of interactions between verbascoside and hCA I(4WR7). Blue color represents hydrogen bondings, grey color represents hydrophobic interactions, and green represents π-stacking interactions.

When hCA II docking results of the compounds were evaluated, it was seen that verbascoside had lower energy score(−10.08 kcal/mol)than oleuropein(−8.58 kcal/mol). Thus, this confirmed the strong interactions between verbascoside and the active site of the 3HS4. The phenyl moiety of verbascoside realized one π-stacking interaction with the imidazole ring of the amino acid His94, as presented in Fig. 6. In addition, the carboxylate group of verbascoside realized a salt bridge with the same amino acid His94, which is one of the amino acids that make metal complexes

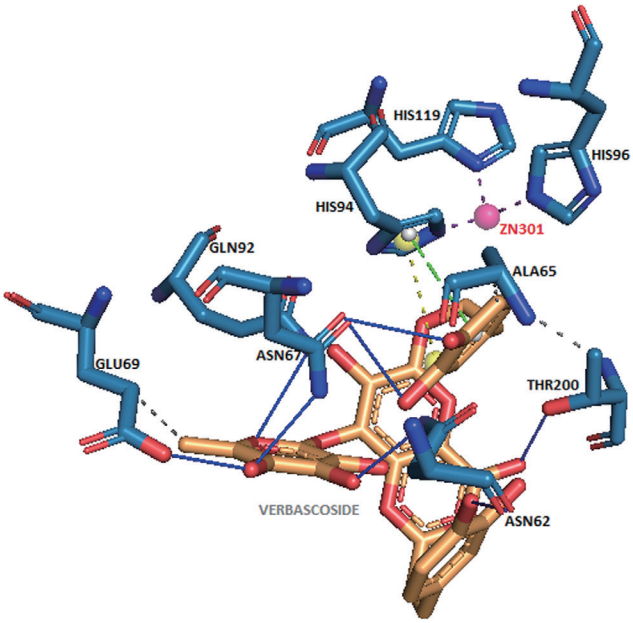

Fig. 6 Schematic presentation of interactions between verbascoside and hCA II(3HS4). Blue color represents hydrogen bondings, grey color represents hydrophobic interactions, green represents π-stacking interactions, and yellow represents salt bridges.

with Zn ion in the active site of the enzyme. This may explain the high inhibitory effect of verbascoside on hCA II isoenzyme. According to Fig. 6, verbascoside interacted by nine hydrogen bonding with the side chains of the amino acids Asn62, Asn67, Glu69, Gln92, and Thr200. On the other hand, verbascoside had three hydrophobic interactions with the amino acids Ala65, Glu69, and Thr200.

When Fig. 7 was evaluated, it was seen that the carboxylate groups and the ose moiety of oleuropein realized two salt bridges with the imidazole rings of the amino acids His64 and His94. In addition, the phenyl moiety of oleuropein realized one π-cation interaction with the imidazole ring of the amino acid His94. According to Fig. 7, the hydroxyl groups of oleuropein realized eight hydrogen bondings with the side chains of the amino acids Trp5, Asn62, His64, Asn67, Gln92, Glu106, and Thr199. On the other hand, the hydroxyl and methylene groups of oleuropein realized three hydrophobic interactions with the amino acids Phe131, Leu198, and Thr200.

4 Conclusion

In conclusion, oleuropein and verbascoside, which are abundant in olive oil, had significant inhibitory potential against hCA I, and II isoenzymes. These polyphenolic and natural compounds showed the effective inhibitory effects

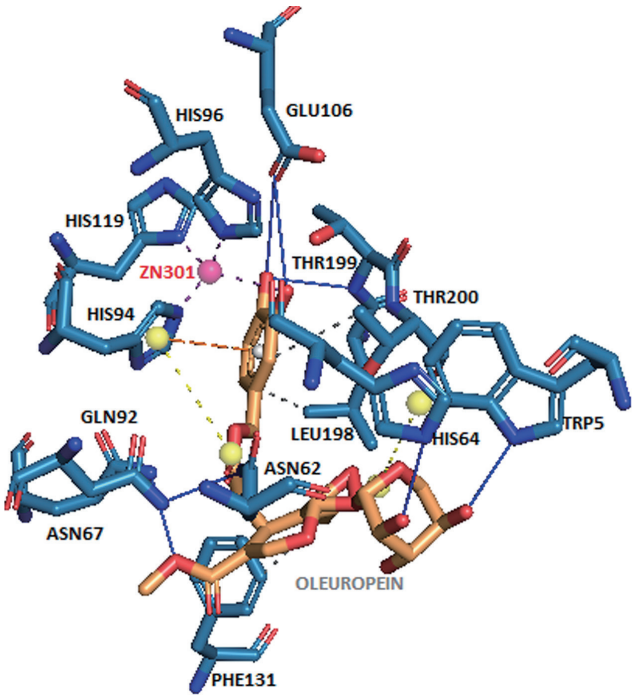

Fig. 7 Schematic presentation of interactions between oleuropein and hCA II(3HS4). Blue color represents hydrogen bondings, grey color represents hydrophobic interactions, green represents π-stacking interactions, and yellow represents salt bridges.

at low micromolar concentrations against both hCA isoenzymes. The results clearly indicate oleuropein and verbascoside may contribute to the development of new drugs in the treatment of many global disorders associated with hCA I, and hCA II isoenzymes.

Author Contributions

IG and AGA conceived the original idea. IG was in charge of overall direction and planning. AGA participated in the study design and coordination, drafted the manuscript. AGA, PT, MK and NU were responsible for the purification of enzyme and determination of inhibition effects of the compounds on hCA I, and II isoenzymes. IG, AGA, and PT contributed to the analysis of the results. Molecular docking studies were carried out by SB. AGA wrote the manuscript with supports from IG and PT. All authors discussed the results and contributed to the final manuscript.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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