A pre-clinical trial study on afzelin: anti-human lung cancer, anti-cholinesterase, and anti-glucosidase properties

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Abstract

Introduction: Afzelin is a glycosyloxyflavone that consists of kaempferol attached to an α-L-rhamnosyl residue at position 3 via a glycosidic linkage. It has a role as a plant metabolite, an antibacterial agent and an anti-inflammatory agent. It is a glycosyloxyflavone, a trihydroxyflavone and a monosaccharide derivative. In this study, we determined the anticholinergic, antiepileptic, antidiabetic, and anti-human lung cancer capacities of afzelin.
Material and methods: IC_{50} values were calculated for both acetylcholinesterase and α-glycosidase as key enzymes affected by afzelin. The cytotoxicity and anticancer potential of afzelin in human lung was evaluated using the common cytotoxicity test, i.e. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in *in vitro* conditions.
Results: IC_{50} values of 365.11 nM for acetyl cholinesterase and 0.94 nM for α-glycosidase were calculated in this study. For these enzymes, Ki values were found to be 300.65 ±56.01 nM for acetyl cholinesterase and 1.65 ±0.11 nM for α-glucosidase. Cell viability of afzelin was very low against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines without any cytotoxicity towards the normal cell line.
Conclusions: Afzelin shows significant antioxidant and anti-human lung cancer properties. It appears that the anti-human lung carcinoma effect of afzelin is due to its antioxidant effects.

Key words: afzelin, acetylcholinesterase, anti-human lung cancer activities. molecular modeling, ADME/T.

Introduction

Afzelin as a natural compound is a flavonol recorded in *Nymphaea odorata* and *Ficus palmata*. Also, it has been recorded to inhibit cyclooxygenase (COX)-1, lipid peroxidation, and COX-2 *in vivo*. The rhamnoside of kaempferol that has been documented to suppress inflammatory cell infiltration in a mouse asthma model [1]. Recently, it was found that afzelin inhibits lung cancer cell development by inducing apoptosis, while being fairly non-toxic to normal cells [2]. Indeed, it remains for the effects of afzelin on asthma phenotypes to be elucidated. The present research

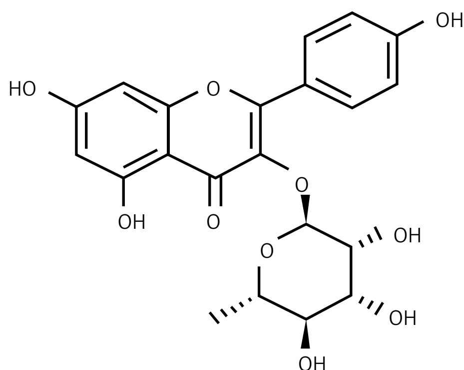

Figure 1. Chemical structure of afzelin

was conducted to investigate the anti-asthmatic effect of afzelin in a mouse model of asthma and its mechanism of action [3] (Figure 1).

Phenolic compounds show physiological properties such as anti-allergic, anti-atherogenic, anti-microbial, anti-inflammatory, antioxidant, anti-thrombotic, and cardiovascular-expanding effects [4–8]. Consumption of phenolic compounds has been reported to be beneficial to health [9, 10]. This beneficial effect is thought to be due to the antioxidant activities of phenolic compounds [11]. Phenolic compounds can be an important determinant of antioxidant potentials of foods [10–12].

Among the various factors implicated in pathogenesis of Alzheimer's disease (AD), decrease of acetylcholine (ACh) as the main neurotransmitter in the brain, formation of amyloid β-peptide (Aβ) plaques and neurofibrillary tangles in the brain, and oxidative stress seem more important than the others. Today, the main drugs to control AD are AChE inhibitors. These drugs increase ACh levels in the brain and improve cognitive function by inhibiting AChE, which normally decomposes ACh [13–17].

α-Glucosidase enzyme inhibitors (e.g. miglitol, acarbose) are extensively used in the therapy of patients with type 2 diabetes (T2D). These inhibitors delay the absorption of carbohydrate molecules from the small intestine and also have a lowering effect on postprandial blood glucose and insulin levels [18]. Acarbose, as one of the most extensively used drugs in the therapy of type 2 diabetes, is a α-glucosidase inhibitor. α-Glucosidase is recorded on hydrolysis of carbohydrate to glucose and brush borders of the intestine and thus its inhibition leads to a decrease in glucose uptake [18].

In recent studies, it has been seen that theoretical calculations are more advanced and developed with developing technology. Among the theoretical methods, the most common and fastest molecular docking method was utilized to compare the biochemical activities of the afzelin molecule against enzymes [19, 20]. In these calculations, the following enzymes are used: α-galactosidase (α-Gly) (PDB ID: 1R47) [21], acetylcholinesterase (AChE) (PDB ID: 4M0E) [22], (PDB ID: 5AML) [22]. Afterwards, ADME/T (metabolism, distribution, absorption, toxicity, and excretion) analysis [23–25] was carried out to examine the drug availability properties of the afzelin molecule. We also investigated afzelin in cytotoxicity studies against common human lung cancer cell lines, i.e., LC-2/ad, PC-14, and HLC-1 cell lines, *in vitro*.

Material and methods

Enzyme studies

AChI was used as reaction substrate molecules and as a previous study, DTNB was used to determine anticholinesterase activity and conducted in accordance with the Ellman method [26]. The inhibition effect of afzelin on glycosidase was assessed using the assay by Tao *et al.* [27] and conforming to previous studies [28].

Molecular docking method

In many studies conducted today, theoretical studies are a significant guide to experimental studies [29]. It is easier to design more active compounds with more parameters obtained through theoretical studies. Preliminary preparation is required for the docking calculations of molecules to be made. At first, the compound was optimized utilizing the Gaussian software assay [30]. Afterwards, molecular modeling calculations were made using these optimized structures. In these calculations, the Maestro Molecular modeling platform (version 12.2) was made using Schrödinger LLC [31]. In the first of these modules, the studied proteins must be prepared for calculations to be made. The protein preparation module [32] is used for this calculation. Enzymes are formed by combining many proteins. These proteins were minimized at first using this module. The compounds in the structure of many proteins that make up the enzyme are removed for molecular docking calculations. At the stage of preparation of enzymes, active regions of proteins were recorded. The protein molecules in this active region were given freedom of movement to interact. As the next module, the LigPrep module [33] was utilized to prepare the compounds for molecular docking calculations. In this module, 3D structures of high-energy isomers at physiological pH are obtained by calculations of molecules. These calculations were made as a result of interactions with these 3D structures with proteins [34–36].

Determination of anti-human lung cancer effects of afzelin

In this assay, different human lung cancer cell lines, i.e., lung poorly differentiated adenocarcino-

ma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines and also a normal cell line (HUVEC) were used to study the cytotoxicity and anticancer potential of afzelin in human lung using the common cytotoxicity test, i.e. the MTT assay in *in vitro* conditions.

15 ml of RPMI 1640 medium containing 10% FSC (10 mg/ml penicillin and 100 mg/ml streptomycin) in a culture flask was placed in a CO_2 incubator for 2 h to equilibrate the medium. Under safe conditions (using insulated gloves and goggles) the frozen cell vial was removed from the nitrogen storage tank. The melting process should be completed in about 1 min and the cells should not be overheated. The medium was added dropwise to the vial and then its contents were taken out and centrifuged with the medium in 15 ml sterile test tubes. After centrifugation, the supernatant was removed and the cells were suspended again in the medium and transferred to a pre-prepared flask containing the medium and FBS and incubated [37].

The cell lines were cultured in RPMI 1640 medium containing penicillin (100 IU/ml), streptomycin (100 IU/ml), glutamine (2 mmol) and 10% fetal bovine serum (FBS). Cells were incubated at 37°C and in an atmosphere containing 0.5 CO_2. Cells began to grow in 75 cm^2 T-flasks in 15 ml of medium with an initial number of 1–2 × 10^6 cells. The cells were then suspended in a fresh culture medium with the help of a Pasteur pipette and the suspension was poured into 100-well plate flat wells (for cell culture) using an 8-channel sampler of 100 µl. Another column was considered to contain culture medium and healthy cells, and other columns were considered to contain culture medium and cell line cells. One of these columns, which contained culture medium and cells and did not contain afzelin, was considered as a control [37].

The plates were incubated in the incubator for 24 h to return the cells to normal from the stress of trypsinization. After this time, suitable dilutions of the prepared afzelin (0–1000 µl/ml) and 100 µl of each dilution were added in columns to the plate wells. (Thus, the final concentration of the studied compound in the wells was halved. Therefore, the concentrations were prepared twice as high to reach the final concentration after being added to the well). The cells were incubated for 37 h at 37°C and 5% CO_2 in the atmosphere. After 72 h, 20 µl of MTT solution (5 mg/ml) was added to each well. The plates were incubated for 3 to 4 h and then the residue was removed and 100 µl of DMSO was added to each well to dissolve the resulting formazan. After 10 min, using shaking of the plates, the optical absorption of formazan at 570 nm was read using a plate reader. Wells containing cells without afzelin were considered as a control and wells without cells and only culture medium were considered as a blank. The percentage of cell viability was calculated using the following formula [37]:

$$\text{Cell viability (\%)} = \frac{\text{Sample A}}{\text{Control A}} \times 100 .$$

Qualitative measurement

After collecting data, Minitab statistical software was used for statistical analysis. Evaluation of cytotoxicity results in a completely randomized design and comparison of means was by the Duncan post-hoc test with a maximum error of 5%. To measure the percentage of cell survival in factorial experiments with the original design of completely randomized blocks and compare the means, the Duncan post-hoc test with a maximum error of 5% was used. The 50% cytotoxicity (IC_{50}) and 50% free radical scavenging (IC_{50})) were estimated with ED50 plus software (INER, V: 1.0). Measurements were reported as mean ± standard deviation.

Results and Discussion

Enzyme results

Inhibition of main enzymes was performed, and also their results were recorded as follows.

Afzelin was effective in inhibiting the AChE enzyme as a type of metabolic enzyme. The K_i value for this enzyme was 300.65 ±56.01 nM (Table I). Also, the galantamine molecule was utilized as an AChE enzyme control molecule; it had a K_i value of 122.21 ±14.04 nM. Afzelin and galantamine IC_{50} values were: afzelin 365.11 nM, bigger than galantamine (148.98 nM) for AChE. On the other hand, afzelin as an α-glycosidase inhibitor in this study showed IC_{50} and K_i values of 0.94 nM and 1.65 ±0.11 (Table I). The results of the α-glycosidase assay showed that afzelin exhibits effective α-glycosidase inhibition compared to acarbose (IC_{50}: 8.81 nM, K_i:11.53 ±2.73) as a control antidiabetic inhibitor [38]. AChE inhibitors are the most promising therapeutics for AD therapy as they prevent ACh loss and slow the progression of the disease. An important drug group utilized in the therapy of AD is cholinesterase inhibitor molecules. The first cholinesterase inhibitor registered for the symptomatic therapy of AD is tacrine. Cholinesterase inhibitors currently on the market are rivastigmine, galantamine, and donepezil and tacrine. In fact, cholinesterase inhibitors are classified as short-acting or reversible factors like donepezil, tacrine, and galantamine, or medium-acting or pseudo-irreversible factors like rivastigmine, according to their mechanism of action. T2DM can be successfully treated with antidiabetic factors that have the ability to delay

Table I. Enzyme inhibition results of afzelin against some enzymes

Compounds	IC_{50} [nM]				Ki	
	AChE	%Inhibition	α-Gly	%Inhibition	AChE	α-Gly
Afzelin	365.11	60.65	0.94	74.08	300.65 ±56.01	1.65 ±0.11
Galantamine	148.98	79.12	–	–	122.21 ±14.04	–
Acarbose	–	–	8.81	58.87	–	11.53 ±2.73

and reduce postprandial blood glucose amounts. α-Glycosidase plays a role in carbohydrate metabolism and has a significant function in cancer, viral infections, and diabetes. This metabolic enzyme has various biochemical activities and is considered an attractive drug target. Recently, a number of α-glycosidase inhibitor compounds have been discovered and also studied. Antidiabetic drugs used in clinical practice, such as voglibose, acarbose, and miglitol, competitively inhibit the metabolic enzyme in the brush border of the small intestine, which interrupts carbohydrate hydrolysis and improves postprandial hyperglycemia [39, 40].

Molecular docking results

Many experimental and theoretical methods are utilized to compare the biochemical activities of the afzelin molecule against enzymes. The fastest and most common of these experimental and theoretical studies is molecular docking. Among the theoretical studies, molecular docking is the method that gives the most compatible results with experimental studies [41].

Molecular placement calculations were compared with the biochemical activities of the afzelin molecule against enzymes. In these calculations performed to compare biological activities, many parameters related to the biochemical activities of the compound were obtained. By comparing the numerical values a lot of information about the molecule is obtained. These parameters are given in Table II. Among these parameters, the most important are the docking score and glide model parameters. These parameters give information about biochemical activity. The compound with the most negative numerical value of these parameters has the highest biochemical activity value. The high biochemical activity of compounds indicates the highest interaction between compounds and proteins. The more interactions among the compound and the proteins in the enzyme, the more the compound binds to the enzyme. These interactions have many interactions including polar, hydrogen bonds, π-π and halogen bonds, and hydrophobic interactions [42]. As a result, this increases the biochemical activity of the compound [43]. These interactions among compounds and studied enzymes are given in Figures 2 and 3.

Table II. Numerical values of docking parameters of molecule against enzymes

Variable	AChE	α-Gly
Docking Score	–9.06	–6.30
Glide ligand efficiency	–0.29	–0.20
Glide hbond	–0.38	–0.32
Glide evdw	–34.78	–27.66
Glide ecoul	–19.28	–15.31
Glide emodel	–87.48	–67.66
Glide energy	–54.06	–42.97
Glide einternal	10.73	6.56
Glide posenum	217	26

As a result of these calculations, after comparing the biochemical activities of the afzelin compound against enzymes, an ADME/T analysis was performed to estimate the effect of the afzelin compound on human metabolism. Among these parameters, the first parameter is dissolved molecular weight, which requires the compound to have a specific MW [44].

Results of anti-human lung cancer effects

The MTT assay is a colorimetric procedure based on reduction and breaking of yellow tetrazolium crystals by the enzyme succinate dehydrogenase to form insoluble purple crystals. In this method, unlike other methods, the steps of washing and collecting cells, which often cause the loss of a number of cells and increase the work error, have been eliminated and all test steps from the beginning of cell culture to reading the results with a photometer are performed on a microplate, so the repeatability, accuracy and sensitivity of the test are high [45]. If the experiment is done on cells linked to the plate, an appropriate number of cells (about 2,000 cells) must first be cultured in each of the wells. Then we select the control and test wells and add the appropriate amount of mitogen or drug to the test wells and place the plate in the incubator for the required time so that the desired substance affects the cells [46]. At the end of the incubation time, the supernatant is discarded and 200 µl of culture medium containing half an mg/ml of MTT solution is added to each well and it is placed again in a carbon dioxide incuba-

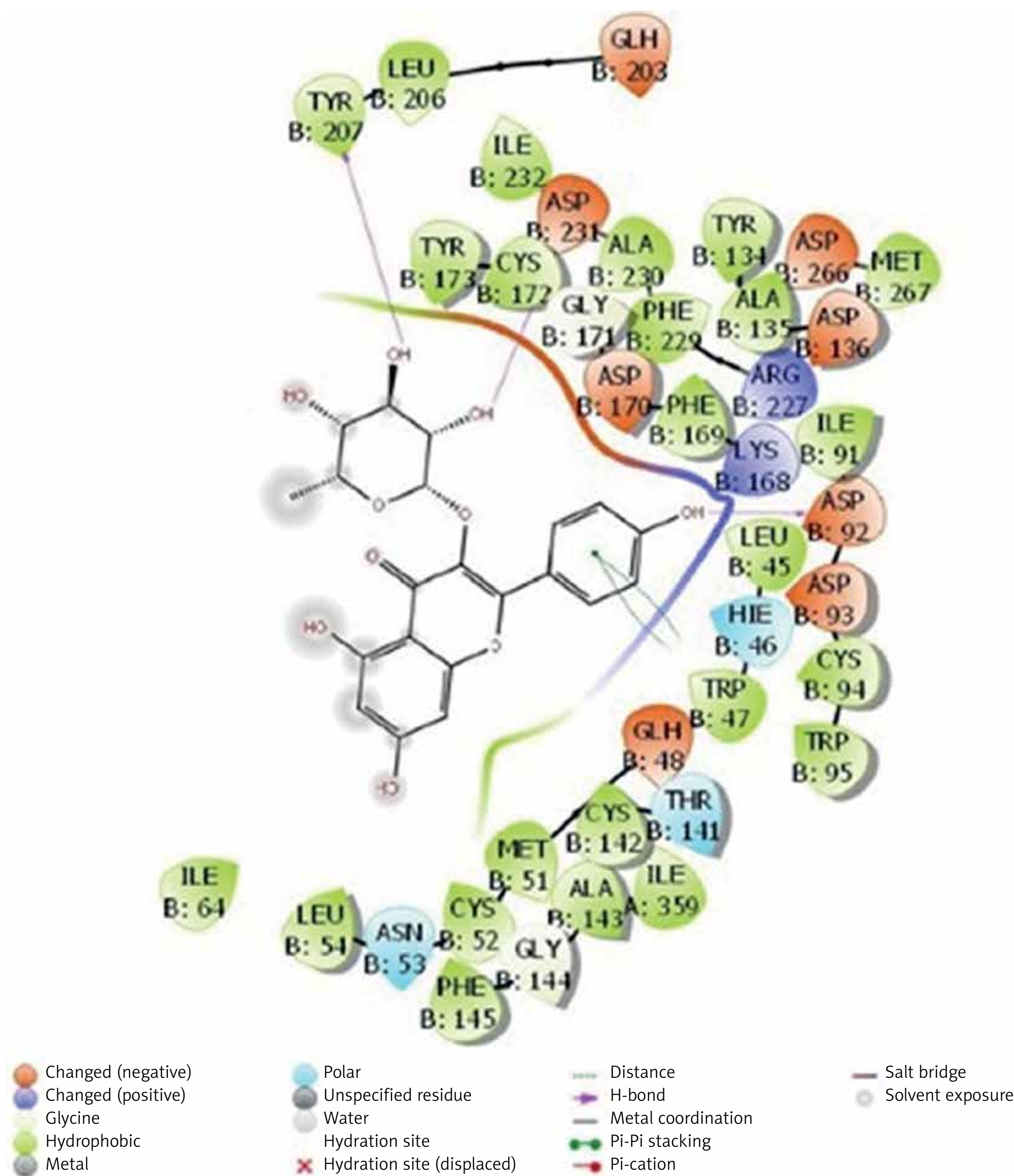

Figure 2. Presentation of interactions of afzelin with AChE enzyme

tor for 2 to 4 h at 37°C. During incubation, MTT is regenerated by one of the enzymes of the mitochondrial respiratory cycle, i.e., succinate dehydrogenase. The regeneration and breakage of this ring produce purple-blue crystals of formazan that are easily detectable under a microscope. At the end, the optical absorption of the resulting solution can be read at 570 nm and the cell number can be calculated using a standard curve [47].

In this present study, the cytotoxicity of afzelin was explored by studying its interaction with HUVEC, LC-2/ad, PC-14, and HLC-1 cell lines by MTT assay for 48 h. The interactions expressed as cell viability (%) were observed at different afzelin concentrations (0–1000 µg/ml) with the four cell lines which are shown in Figure 4. In all the cases the % cell viability decreases with increasing afzelin concentrations. The IC_{50} values of afzelin against LC-2/ad, PC-14, and HLC-1 cell lines were 394, 427, and 581 µg/ml, respectively (Table III).

Oxidation from reactive oxygen species can cause cell membrane disintegration, damage to membrane proteins, and DNA mutation, and the result is the onset or exacerbation of many diseases such as cancer, liver damage, and cardiovascular disease. Although the body has a defense system, constant exposure to chemicals and contaminants can lead to an increase in the number of free radicals outside the body's defense capacity and irreversible oxidative damage [46]. Therefore, antioxidants with the property of removing free radicals play an important role in the preven-

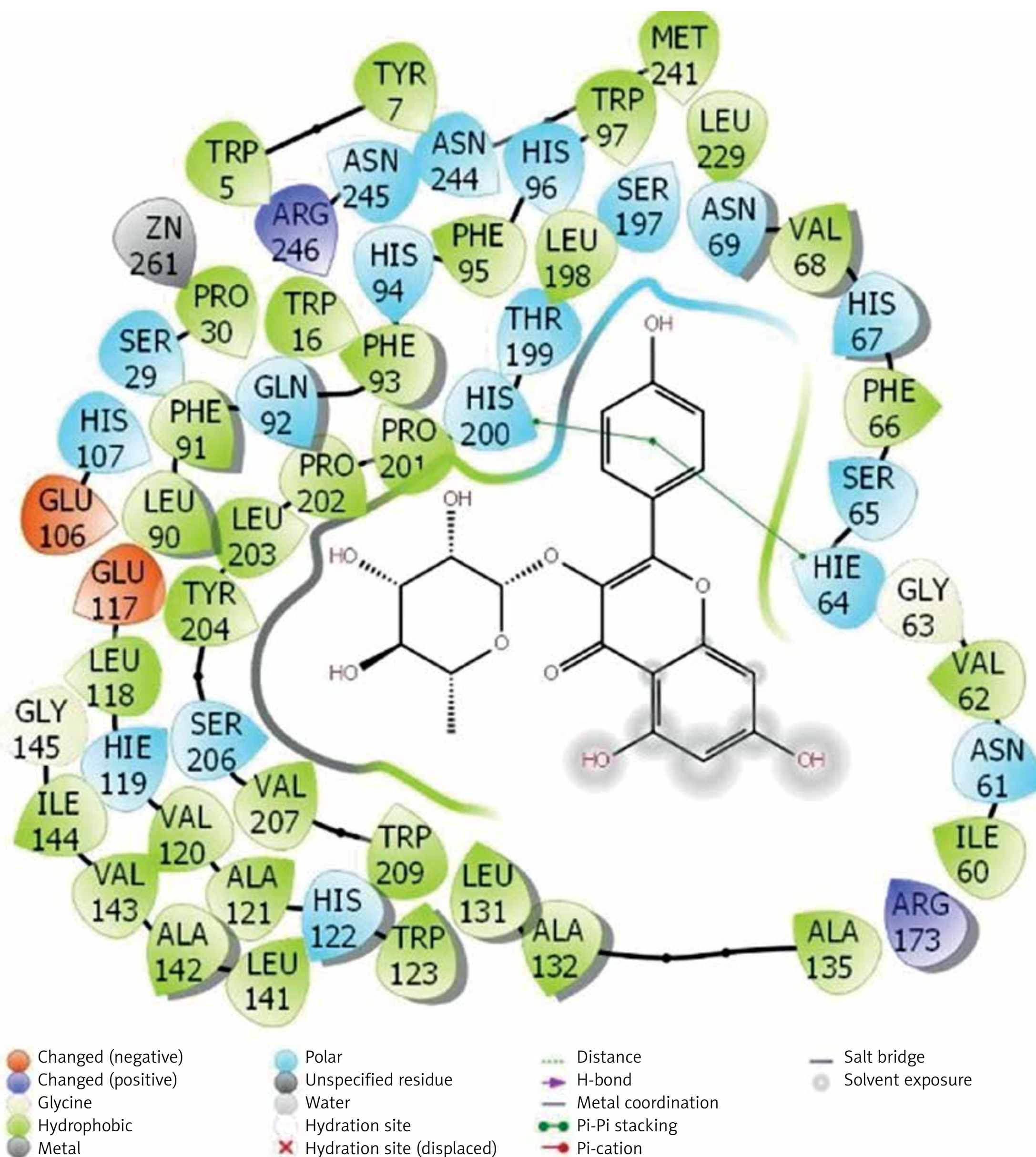

Figure 3. Presentation of interactions of afzelin with α-Gly enzyme

tion or treatment of oxidation-related diseases or free radicals. Extensive molecular cell research on cancer cells has developed a targeted approach to the biochemical prevention of cancers the goal of which is to stop or return cells to their pre-cancerous state without any toxic doses through nutrients and drugs. Numerous studies have been performed on the use of natural compounds as anti-cancer agents in relation to appropriate antioxidant activity [47]. It seems the high anti-lung adenocarcinoma properties of afzelin are related to its antioxidant activities.

In conclusion, due to the advantages of antioxidant molecules such as the ability to carry drugs, reduce toxicity, controlled drug release and specific drug delivery to the target tissue, these structures have attracted the attention of many researchers. As a result of these features, antioxidant molecules have great potential for cancer treatment that can move from a research laboratory to a patient's bedside. One possible concern that limits the administration of some antioxidant molecules in treating cancer is their toxicity, which needs further investigation. However, antioxidant molecule-based cancer therapies will continue to be developed to improve treatment outcomes. In this research, afzelin was first assayed to inhibit AChE and α-GLY. The inhibitory activity was more intense versus AChE compared to the standard inhibitors (ACR – acarbose), which, in turn, displayed stronger inhibition with respect to α-GLY. Also, afzelin was detected to have a high selective index

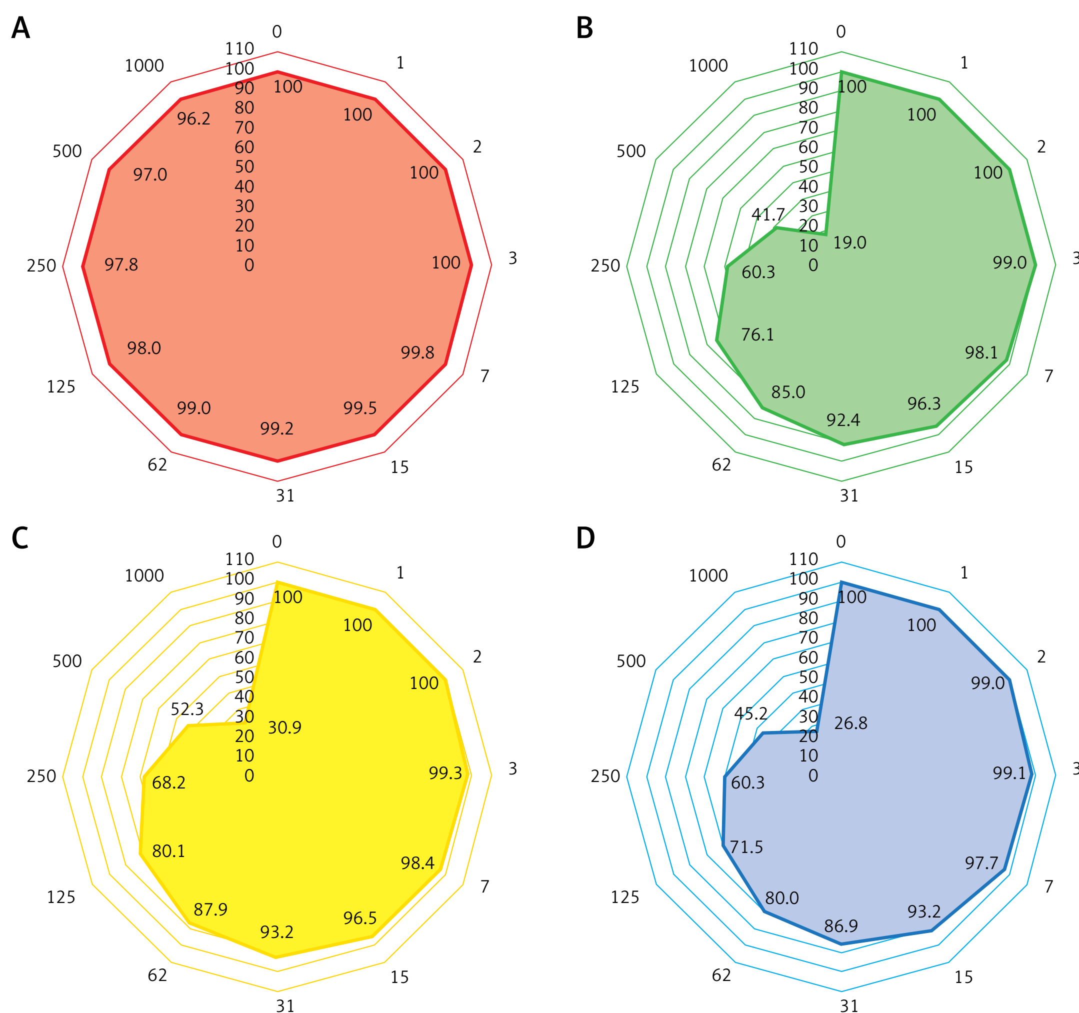

Figure 4. The anti-human lung cancer properties (cell viability (%)) of afzelin (concentrations of 0–1000 μg/ml) against HUVEC (**A**), lung poorly differentiated adenocarcinoma (PC-14: **B**), lung well-differentiated bronchogenic adenocarcinoma (HLC-1: **C**), and lung moderately differentiated adenocarcinoma (LC-2/ad: **D**) cell lines. The numbers indicate the percent of cell viability in the concentrations of 0–000 μg/ml of afzelin against several human lung cancer cell lines

Table III. IC_{50} of afzelin in the anti-human lung cancer test

	HUVEC	PC-14	HLC-1	LC-2/ad
IC_{50} [μg/ml]	–	394 ±0	581 ±0	427 ±0

towards AChE. It also showed significant cytotoxic activities against common human lung cancer cell lines, i.e. LC-2/ad, PC-14, and HLC-1 cell lines.

Conflict of interest

The authors declare no conflict of interest.

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