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

Type
Research paper

Keywords
Afzelin, Acetylcholinesterase, Anti-human lung cancer activities. Molecular modeling, ADME/T

Abstract
Introduction
Afzelin is a glycosyl付款 flavone that is kaempferol attached to an alpha-L-rhamnosyl residue at position 3 via a glycosidic linkage. It has a role as a plant metabolite, an antimicrobial (Antibacterial and antifungal) agent and an anti-inflammatory agent. It is a glycosyloxyflavone, a trihydroxyflavone and a monosaccharide derivative. In this work, we determined the anticholinergic, antiepileptic, antidiabetic, and anti-human lung cancer capacities of afzelin.

Material and methods
IC50 values were calculated for both acetylcholinesterase, and α-glycosidase as key enzymes effects of afzelin. The cytotoxicity and anticancer potential of human lung over the Afzelin using the common cytotoxicity test i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in vitro condition.

Results
IC50 values obtained 365.11 nM for acetyl cholinesterase and 0.94 nM for α-glycosidase were calculated in this study. Indeed, for these enzymes, Ki values were found as 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 on the normal cell line.

Conclusions
As mentioned, the Afzelin had significant antioxidant and anti-human lung cancer properties. It appears that the anti-human lung carcinoma effect of Afzelin is due to their antioxidant effects.
A pre-clinical trial study on Afzelin: Anti-human lung cancer, anti-cholinesterase, and anti-glucosidase properties

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ABSTRACT

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Keywords: Afzelin, Acetylcholinesterase, Anti-human lung cancer activities. Molecular modeling, ADME/T
1. INTRODUCTION

Afzelin as a natural compound is a flavonol which is recorded in Nymphaea odorata and Ficus palmata. Also, it has been recorded to inhibit cyclooxygenase (COX)-1, lipid peroxidation, and COX-2 in vivo. It is kaempferol's rhamnoside 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 remained for the effects of afzelin on asthma phenotypes to be elucidated. The present research was conducted to investigate the anti-asthmatic effect of afzelin in a mouse model of asthma and its mechanism of action (3).

![Figure 1. The chemical structure of afzelin](image)

Phenolic compounds show physiological properties such as anti-allergic, anti-atherogenic, anti-microbial, anti-inflammatory, antioxidant, antithrombotic, cardiovascular-expanding effect (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 reasons that mentioned for pathogenesis of Alzheimer's disease (AD), decrease of acetylcholine (ACh) as main neurotransmitter in 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 that normally decomposes ACh (13-17).

α-Glucosidase enzyme inhibitors (like miglitol, acarbose) are extensively used in the therapy of patients with 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 hydrolyzed carbohydrate to glucose and brushy surface of the intestine and thus its inhibition leads to a decrease in glucose uptake (18).

Theoretical studies are developing more and more every passing day. 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 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 (33,34) was carried out to examine the drug availability properties of Afzelin molecule. We also investigated the Afzelin in the cytotoxicity studies against common human lung cancer cell lines i.e., LC-2/ad, PC-14, and HLC-1 cell lines, in-vitro.

2. EXPERIMENTAL

2.1. Enzymes 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 Tao et al assay (27) and conforming to previous studies (28).

2. 3. 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, compound was optimized utilizing the Gaussian software assay (30). Afterwards, molecular modeling calculations were made using these optimized structures. In these calculations, Maestro Molecular modeling platform (version 12.2) was made by using Schrödinger, LLC (31). In the first module between 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).

2.4 Determination of anti-human lung cancer effects of Afzelin

In this assay, different human lung cancer cell lines i.e., lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines and also the normal cell line (HUVEC) were used to study the cytotoxicity and anticancer potential of human lung over the Afzelin using the common cytotoxicity test i.e., MTT assay in vitro condition.

15 ml of RPMI 1640 medium containing 10% FSC (10 mg/ml penicillin and 100 mg/ml streptomycin) in a culture flask, placed in a CO2 incubator for 2 hours to equilibrate the medium. Under safe conditions (using insulated gloves and goggles) the frozen cell vial was removed from the nitrogen storage tank. In order to avoid the possibility of explosion of the vial (due to the possible entry of liquid nitrogen into the vial), loosen the lid, after disinfecting the outer surface of the vial with 70% alcohol, under the hood to remove nitrogen gas. Close the vial lid again and immediately melt it in a pan at 37 °C. The melting process should be completed in about 1 minute and the cells should be avoided from overheating. The medium was added dropwise to the vial and then its contents were taken out and centrifuged with the medium in 15 cc 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).

Cell line used in RPMI 1640 medium containing penicillin (100 IU / ML), streptomycin (100 IU / ML), glutamine (2 mmol) and 10% fetal bovine serum (FBS). They were incubated at 37 °C and in an atmosphere containing 0.5 CO2. Cells began to grow in 75 cm² T-flasks in 15 ml medium with an initial number of 1-2 × 10⁶ cells. After three days
and covering the flask bed with the cell, the adhesive layer to the bottom of the flask was
separated enzymatically using trypsin-versen and transferred to a sterile test tube for 10
minutes at 1200 rpm. 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. One column of wells was kept cell-free
and as a plank containing only culture medium. In another column, it was considered to
contain culture medium and healthy cells and in other columns, it was 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 hours 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 columnarly to the plate wells (Thus,
the final concentration of the studied compound in the wells was halved. Therefore, the
concentrations were prepared twice as much to reach the final concentration after being
added to the well). The cells were incubated for 37 hours at 37 ° C and 5% CO2 in the
atmosphere. After 72 hours, 20 µl of MTT solution (5 mg/ml) was added to each well. The
plates were incubated for 3 to 4 hours and then the residue was removed and 100 µl of
DMSO was added to each well to dissolve the resulting formazan. After 10 minutes, using
shaking the plates, the optical absorption of Formazan at 570 nm was read using a plate
reader. Wells containing cells without Afzelin were considered as control and the optical
density of wells without cells and only culture medium were considered as blank. The
percentage of cell viability was calculated using the following formula [37]:

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

2.5 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 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, Duncan post-hoc test with a maximum error of 5% was
used. The 50% cytotoxicity (IC50) and 50% free radical scavenging (IC50)) was estimated with ED50 plus software (INER, V: 1.0). Measurements were reported as mean ± standard deviation.

3. RESULT AND DISCUSSION

3.1. Enzymes Results

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

Afzelin was effective inhibiting AChE enzyme as a type of metabolic enzyme. Kᵢ value for this enzyme was obtained to be 300.65±56.01 nM (Table 1). Also, the galantamine molecule was utilized as AChE enzyme control molecule; it had Kᵢ value of 122.21±14.04 nM. Afzelin and galantamine values IC₅₀ were: Afzelin (365.11 nM) bigger than galantamine (148.98 nM) for AChE. On the other hand, Afzelin as α-glycosidase inhibitor in this study shown as IC₅₀ and Kᵢ values are 0.94 nM, and 1.65±0.11 (Table 1). The results of the α-glycosidase assay showed that Afzelin has an effective α-glycosidase inhibition compared to acarbose (IC₅₀: 8.81 nM, Kᵢ: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. The important drug group utilized in the therapy of AD are 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, medium-acting or pseudo-irreversible factor-like rivastigmine, according to their mechanism of action. T2DM can be successfully treated with antidiabetic factors that have the ability to delay 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 aim. Currently, a number of α-glycosidase inhibitor compounds have been discovered and also studied. Antidiabetic drugs used in clinical practice, like voglibose, acarbose, and miglitol, competitively inhibit the metabolic enzyme in the brush edge of the small intestine, which interrupts carbohydrate hydrolysis and improves postprandial hyperglycemia (39,40).
Table 1. The enzyme inhibition results of Afzelin against some enzymes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (nM) AChE</th>
<th>%Inhibition</th>
<th>IC₅₀ (nM) α-Gly</th>
<th>%Inhibition</th>
<th>Ki AChE</th>
<th>α-Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afzelin</td>
<td>365.11</td>
<td>60.65</td>
<td>0.94</td>
<td>74.08</td>
<td>300.65±56.01</td>
<td>1.65±0.11</td>
</tr>
<tr>
<td>Galantamine</td>
<td>148.98</td>
<td>79.12</td>
<td>-</td>
<td>-</td>
<td>122.21±14.04</td>
<td>-</td>
</tr>
<tr>
<td>Acarbose</td>
<td>-</td>
<td>-</td>
<td>8.81</td>
<td>58.87</td>
<td>-</td>
<td>11.53±2.73</td>
</tr>
</tbody>
</table>

3.2. Molecular Docking Result

Many experimental and theoretical methods are utilized to compare the biochemical activities of 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 made to compare biological activities, many parameters related to the biochemical activities of the compound were obtained. By comparing the numerical values had a lot of information about the molecule is obtained. These parameters are given in Table 2. Among these parameters, the most important parameter is 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 like 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.

**Figure 2.** Presentation interactions of afzelin with AChE enzyme

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

**Table 2.** Numerical values of the docking parameters of molecule against enzymes

<table>
<thead>
<tr>
<th></th>
<th>AChE</th>
<th>α-Gly</th>
<th>hCA I</th>
<th>hCA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docking Score</td>
<td>-9.06</td>
<td>-6.30</td>
<td>-6.97</td>
<td>-6.89</td>
</tr>
<tr>
<td>Glide ligand efficiency</td>
<td>-0.29</td>
<td>-0.20</td>
<td>-0.22</td>
<td>-0.22</td>
</tr>
<tr>
<td>Glide hbond</td>
<td>-0.38</td>
<td>-0.32</td>
<td>-0.30</td>
<td>-0.60</td>
</tr>
</tbody>
</table>
As a result of these calculations, after comparing the biochemical activities of the afzelin compound against enzymes, an ADME/T 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).

### 3.3. Anti-human lung cancer effects results

The MTT assay is a procedure of colorimetric based on reducing 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, discard the supernatant and add 200 μl of culture medium containing half an mg/ml of MTT solution to each well and put it again in a carbon dioxide incubator for 2 to 4 hours 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 cells 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 48h. The interactions being expressed as cell viability (%) was observed at different Afzelin concentrations (0-1000 μg/mL) with the four cell lines which have been shown in Figure 4. In all the cases the % cell viability

| Glide evdw | -34.78 | -27.66 | -30.65 | -29.96 |
| Glide ecol | -19.28 | -15.31 | -14.75 | -17.51 |
| Glide ecom | -87.48 | -67.66 | -67.46 | -75.71 |
| Glide energy | -54.06 | -42.97 | -45.40 | -47.47 |
| Glide einternal | 10.73 | 6.56 | 5.88 | 3.91 |
| Glide posenum | 217 | 26 | 221 | 372 |
gets reduced with increasing Afzelin concentrations. The IC$_{50}$ values of Afzelin against LC-2/ad, PC-14, and HLC-1 cell lines were found 394, 427, and 581 µg/mL, respectively (Table 3).

Oxidation from reactive oxygen species can cause cell membrane disintegration, damage to membrane proteins, and DNA mutation that 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 prevention 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 that the goal 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.
Fig. 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 0-1000 µg/mL of Afzelin against several human lung cancer cell lines.

Table 3. The IC$_{50}$ of Afzelin in the anti-human lung cancer test.

<table>
<thead>
<tr>
<th></th>
<th>HUVEC</th>
<th>PC-14</th>
<th>HLC-1</th>
<th>LC-2/ad</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ (µg/mL)</td>
<td>-</td>
<td>394±0a</td>
<td>581±0b</td>
<td>427±0a</td>
</tr>
</tbody>
</table>
CONCLUSIONS

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 been able to attract 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 molecules-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 standard inhibitors TAC and ACR, which, in turn, displayed an inhibition stronger with respect to α-GLY. Also, Afzelin was detected towards AChE with a high selective index towards the 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.

Reference


