In Silico Studies, Biological Activities, and Anti-human Pancreatic Cancer Potential of 6-Hydroxy-4-methylcoumarin and 2,5-Dihydroxyacetophenone as Flavonoid Compounds

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Abstract: Coronavirus is one of the RNA viruses with the largest genome; It is a group of viruses known to infect humans very little until the end of the 20th century, generally causing infection in animals (bird, cat, pig, mouse, horse, bat). It is the causative agent of 15-30% of seasonal lower and upper respiratory tract infections, and may rarely cause gastrointestinal and nervous system infections. We have obtained results for the collagenase and elastase enzymes were at the micromolar level. We obtained IC₅₀ results for the collagenase enzyme for 6-hydroxy-4-methylcoumarin 257.22 ± 34.07 µM and for 2,5-dihydroxyacetophenone 74.46 ± 8.61 µM. 6-Hydroxy-4-methylcoumarin and 2,5-dihydroxyacetophenone were considered good inhibitors for elastase enzyme. Additionally, these compounds significantly decreased human pancreatic cancer cell viability from low doses. In addition, 100 µM dose of all compounds caused significant reductions in human pancreatic cancer cell viability. IC₅₀ results (IC₅₀: 10-50 µM) were better than control. In the otherwords, the docking results suggest that both compounds tend to have lower efficacy on the main protease targets of SARS-CoV-2 than standard compounds, (NL-1 and NL-2). The reason for this is that the standard compounds interact strongly and more frequently with the target proteins, and the surface areas they cover on the active surface are much larger than the small ligand molecules studied.

Key words: 6-hydroxy-4-methylcoumarin, 2,5-dihydroxyacetophenone, anti-pancreatic, molecule docking, enzyme inhibition

1 Introduction

Flavonoid compounds are the most popular class of polyphenolic molecules in the human diet including vegetables, fruits, nuts, tea, plant-derived beverages, and wine. These molecules have been recorded to possess an extensive range of bio-activities¹, ². Structural variations of these flavonoids are associated with many different biological and pharmacological activities including antioxidant, anticancer, anti-inflammatory, antihyperglycemic, antidiabetic, antibacterial, antifungal, and antiviral activities. Antioxidant enzymes as well as non-enzymatic antioxidants are the first line of defense against oxidative stress. This oxidative stress is the underline mechanism for diabetic complications³, ⁴. Recently, the high therapeutic properties of these compounds have brought attention of chemists to synthesize diverse types of their analogs by improving the existing synthetic methodologies. It is therefore, the aim of present study was to synthesis and characterize the novel flavonoids and their derivatives. 2,5-Dihydroxyacetophenone molecule is a key compound obtained from Radix rehmanniae preparata, which is extensively used as a herbal medicine in many countries. DHAP has been recorded to possess anti-anxiety, anti-inflammatory, and neuroprotective qualities (Fig. 1)⁵, ⁶.

The collagenase enzyme is an enzyme belonging to the hydrolase class and breaks down the triple helix structure...
of collagen. Hydrolases provide hydrolysis reactions, that is, the destruction of molecules, with the help of $\text{H}^+$ and OH ions of water. The enzyme, named after its substrate, is also known as matrix metalloprotease-1 or metallopeptidase-1. The weights of collagenases vary between 50-60 kDa, their cofactor is Zn metal. One group of proteases, which are extracellular proteolytic enzymes, need $\text{Zn}^{2+}$ or $\text{Ca}^{2+}$ ions in the bound state for their activation, while the other group is serine proteases containing reactive serine in their active sites. Degradation of matrix proteins such as lamin, collagen and fibronectin by metalloproteases and serine proteases facilitate cell migration. Collagenase is one of these enzymes. These enzymes play a key role in physiological conditions such as normal structuring of tissues and systems, wound healing, tissue remodeling and normal development processes, and in pathological processes such as tumor cells spreading to surrounding tissues and disrupting their functions. Elastases, an important enzyme belonging to the chymotrypsin family of serine proteases, are responsible for the fragmentation of extracellular matrix proteins such as elastin and collagen, which are responsible for skin elasticity and strength. The increase of elastase enzyme leads to deterioration of extracellular matrix components, changes in the structure of elastic fibers and consequently it leads to reduction of elastic properties of the skin and formation of wrinkle. In addition, the irregular and excessive release of elastase enzymes cause abnormal distortions in healthy tissues and this causes chronic wounds and inflammatory diseases. The development of elastase enzyme inhibitors due to these different effects is regarded as beneficial for protecting skin elasticity and controlling elastase-associated diseases.

Pancreatic ductal adenocarcinoma is the fifth leading cause of cancer death in the Western world, with an overall 5-year survival rate of less than 1% and a median survival of 4 months after diagnosis. Histologically, cancer cells exhibit poorly differentiated ductal-like structures, often surrounded by an extensive desmoplastic reaction and infiltration by inflammatory cells. The adjacent pancreatic parenchyma harbors sites of acinar cell degeneration and ductal cell proliferation. A high percentage of these cancers overexpress a number of growth factors and their receptors, including EGF, transforming growth factor (TGF)-$\alpha$, CRIPTO, TGF-$\beta$1, epidermal growth factor receptor, acidic FGF, basic fibroblast growth factor, and FGF5. Overexpression of these mitogenic growth factors may contribute to the bioaggression of pancreatic cancers and the abundant stroma formation that is characteristic of this malignancy.

The aim of this study is to examine the potentials of anti-collagenase, anti-elastase, anti-pancreatic cancer, and anti-Coronavirus disease (COVID-19) by using two important compounds and to detect them by molecular modeling.

2 Experimental

2.1 Enzymes

0.05 mL was taken from the prepared sample solutions. On top of it, 0.05 mL of elastase enzyme (0.16 U/mL) was added. Then, 0.9 mL of tris hydrochloride (Tris-HCl) buffer solution of 0.2 M (pH = 7.8) was added to the sample solutions. It was prepared by adding 0.2 M (pH = 7.8)/0.9 mL Tris-HCl buffer solution to 0.1 mL of elastase enzyme solution as a control solution. The blank solution was prepared by adding 0.2 M (pH = 7.8)/0.9 mL Tris-HCl buffer solution to 0.1 mL distilled water. Blank, control and sample solutions were incubated at 37°C for 15 minutes. After incubation, 5 mM 0.05 mL N-Succinyl-Ala-Ala-Ala-p-nitroanilide (STANA) substrate was added to the blank, control and sample solutions and incubated at 37°C for 30 minutes.

The absorbance values of the sample and control solutions against the blank were read at 410 nm. In the study, the anti-elastase inhibition activity values of the samples prepared at different concentrations were calculated. Experiments were repeated 3 times and averaged. The % inhibition values on elastase enzyme of eperezolid-like compounds synthesized for the first time were calculated. The IC$_{50}$ value (the concentration required to inhibit 50% of the activity) was calculated from the regression equation obtained from the linear segment of the curve drawn by applying the concentration to absorbance, % elastase enzyme inhibition data to the ordinate.

Modified inhibitory effect on collagenase enzyme Thring et al. (2009) was determined spectrophotometrically using the method. 50 $\mu$L of the solution containing 0.8 U/mL collagenase was taken, 50 $\mu$L of plant extracts and chemical substance solutions at different concentrations prepared on it were added. This method was performed according to previous studies. The absorbance values of the sample solutions and control solution were read at 340 nm in the UV spectrophotometer against the blank. Experiments were repeated 2 times. The IC$_{50}$ value, which is the amount of substance required for the collagenase enzyme to have a 50% inhibition effect, was calculated with the regression equation obtained from the linear section of the curve drawn by applying the concentration to the absorbance in the graph and the % enzyme inhibition data to the ordi-
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2.2 Molecular docking of different targets (Collagenase, Elastase, and Major protease (mpro) of SARS-CoV-2) with the studied two molecules

The docking of 6-hydroxy-4-methylcoumarin (1) and 2,5-dihydroxyacetophenone (2) as ligands with Collagenase, Elastase, and Major protease (mpro) of SARS-CoV-2 was analyzed using AutoDock Vina[41]. The ligands were drawn and optimized at DFT/B3LYP/6-31G* basis set using Gaussian 09 (G09)[25]. The crystal structures of targets, Collagenase (pdb: 4AR1), Elastase (pdb: 1MCV) and the main protease (Mpro, pdb: 6LU7[26] and 2GTB[27]) structures were downloaded from the protein data bank (http://www.rcsb.org) were used for docking processes. Moreover, these target proteins were prepared and minimized until the root mean square deviation (RMSD) reaches the lower value of 0.05 kcal/mol Å² using Discovery Studio (DS)[3][3]. Define and edit binding site tool of DS was exerted to detect binding site of aforementioned targets against 1 and 2 ligands. AutoDock Vina[41] was employed to predict the conformations and binding interactions for the ligands. The molecular docking of all of the ligands with Collagenase, Elastase, and Major protease (mpro) of SARS-CoV-2 proteins were analyzed, where the respective targets were constant and the ligands were flexible. The best orientation for each complex was selected based on RMSD and the predicted binding energy of the ligands. Further, cluster analysis, based on RMSD values, was used and the most populated cluster with the lowest energy conformation was noted as an authenticated answer.

2.3 Cancer study

2.3.1 Replication of cells

In order to determine the cytotoxic activity of the compounds, human pancreatic cancer cell line was obtained from the American Type Culture Collection (ATCC) and used in the study. Cells were fed twice a week and cell flasks were incubated at 37°C (Thermo Forma II CO2 Incubator, USA) in a 5% CO2 environment throughout the experimental period. Confused cells were removed with trypsin-EDTA solution and counted under the microscope after staining with 0.4% trypan blue. For experimental studies, 96-well plates were seeded with approximately 15 × 10⁴ cells per well[30].

2.3.2 Treatment with test compounds

1-100 µM concentrations of the compounds to be tested were added to the cell seeded wells, and then the plates were incubated for 24 hours at 37°C in 5% CO2 incubator. The possible effects of the applied compounds on cell viability at the end of the incubation were determined by the MTT method[30].

2.3.3 MTT method

MTT solution at a concentration of 0.5 mg/mL was prepared for the analysis of viability levels in cells after compound administration. After the application, 50 µL of MTT solution was added to each well and incubated in a CO2 incubator for 3 hours. After incubation, the solution in the wells was withdrawn and 100 µL of DMSO was added to them. The optical density of the cells in the wells was read in an ELISA plate reader (Thermo MultiskanGo, USA) at a wavelength of 570 nm. The absorbance values obtained from the control wells were averaged and this value was evaluated as 100% cell viability. The absorbance values obtained from the compound treated wells were proportioned to the control absorbance value and the percent viability values were calculated[31].

3 Results

3.1 Enzymes

Inhibition of elastase and collagenase enzymes have been one of the main targets in cosmetic industry for researches to find new antiwrinkle and skin-lightening compounds or extracts particularly with rich polyphenol contents as well as to prove their effectiveness[32].

We have obtained results for the collagenase and elastase enzymes were at the micromolar level. We obtained IC₅₀ results for the collagenase enzyme for 6-hydroxy-4-methylcoumarin 257.22 ± 34.07 µM and for 2,5-dihydroxyacetophenone 74.46 ± 8.61 µM. 6-Hydroxy-4-methylcoumarin and 2,5-dihydroxyacetophenone were considered good inhibitors for elastase enzyme, and their IC₅₀ results were 63.18 ± 2.35 and 28.66 ± 4.41 µM, respectively. Additionally, We obtained IC₅₀ result for the collagenase as control compound with 101.37 µM and for elastase 30.55 µM. 2,5-Dihydroxyacetophenone was recorded good inhibitor for both enzymes when compared to control, and their IC₅₀ results of collagenase and elastase enzymes were 74.46 and 28.66 µM, respectively (Table 1).

3.2 Molecular docking results

The compound 1 and 2 in this research exhibited affinity to Collagenase, Elastase, and Major protease (mpro) of SARS-CoV-2, with a minimum energy requirement. Among the 200 different conformers of each ligand tested for the inhibition of the related target, compound 2 displayed the potential for a high inhibition property based on the minimum energy of the ligand (−7.35 kcal/mol, and −7.10 kcal/mol in Table 2 and Fig. 2), to comply into the binding site of Collagenase and Elastase models. In vitro assay in this research has shown that administration of 6-hydroxy-4-methylcoumarin (1) and 2,5-dihydroxyacetophenone (2), inhibited Collagenase and Elastase activities based on the standards, Phosphoramidon and N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone, respectively.

Besides these, compound 2 forms three hydrogen bonds
with Glu430, Tyr428 and Gln462 in the binding site of collagenase; and also three hydrophobic interactions with His459 and Trp471 amino acids of aforementioned target Fig. 3. In the meantime, the same compound has four H-bonds with Cys191, Gly193, and Gly192 and one hydrophobic interaction with Val216 residue of elastase. These interactions of compound 2 against each target were represented in Fig. 4. Another one, compound 1 has three hydrogen bonds (Gly463, Gln462 and Gln486), six hydrophobic interactions (Trp471, His459 and Trp471) with collagenase, (Fig. 3). For elastase protein, compound 1 occurs two H-bonds with Asp194, Ser195; one \( \pi \)-lone pair bond with Ser195; and two hydrophobic interactions with His57 and Val216 residues of the target, as given in Fig. 4.

On the other side, the ligand molecule, 6-hydroxy-4-methylcoumarin (1) was found to interact with the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Enzymes results of both compounds.</th>
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<tbody>
<tr>
<td><strong>NO</strong></td>
<td><strong>Compounds</strong></td>
</tr>
<tr>
<td>1</td>
<td>6-Hydroxy-4-methylcoumarin</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2,5-Dihydroxyacetophenone</td>
</tr>
<tr>
<td></td>
<td>Phosphoramidon*</td>
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</tbody>
</table>

\( N-(\text{Methoxysuccinyl})-\text{Ala-Ala-Pro-Val-chloromethyl ketone} \)

\( \) _They are standards_

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The free binding energy value of the compounds, (1-2) with the target enzymes, respectively.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenase</strong></td>
<td><strong>Free Energy of Binding (kcal/mol)</strong></td>
</tr>
<tr>
<td>1</td>
<td>– 6.78</td>
</tr>
<tr>
<td>2</td>
<td>– 7.35</td>
</tr>
<tr>
<td><em>Phosphoramidon</em></td>
<td>– 6.74</td>
</tr>
<tr>
<td><strong>Elastase</strong></td>
<td><strong>Free Energy of Binding (kcal/mol)</strong></td>
</tr>
<tr>
<td>1</td>
<td>– 5.44</td>
</tr>
<tr>
<td>2</td>
<td>– 7.1</td>
</tr>
<tr>
<td>*N-(\text{Methoxysuccinyl})-\text{Ala-Ala-Pro-Val-chloromethyl ketone}</td>
<td>– 6.19</td>
</tr>
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</table>

**Main protease (MP-1)**

| **Free Energy of Binding (kcal/mol)** |
| 1       | – 5.83                                      |
| 2       | – 5.20                                      |
| *NL-1   | – 8.65                                      |

**Main protease (MP-2)**

| **Free Energy of Binding (kcal/mol)** |
| 1       | – 6.32                                      |
| 2       | – 5.76                                      |
| *NL-2   | – 8.41                                      |

* Phosphoramidon (as standard for Collagenase), N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (as standard for Elastase) and NL-1 ((phenylmethyl) \( 4-(\{S\})-4-[[2-(\{S\})-4-methyl-2-[[2-(\{S\})-3-methyl-2-[[2-(\{S\})-2-[[5-methyl-1,2-oxazol-3-yl]carbonylamino]propanoyl]amino]butanoyl]amino]pentanoyl]amino]-5-[[3-(\{S\})-2-oxidanylidenepyrrrolidin-3-yl] pent-2-enoate) for the main protease (Mpro, pdb id: 6LU7) and NL-2 (ethyl(2S)-4-[[3-amino-3-oxo-propyl]-[[2S]-2-[[2S]-4-methyl-2-phenylmethoxycarbonylamino-pentanoyl] amino]-3phenyl-propanoyl] amino]amino]-2-hydroxy-4-oxo-butanoate) for the main protease (Mpro, pdb id: 2GTB).
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Fig. 2  The optimized compound 1, 2, std-1 and std-2 at DFT/B3LYP/6-311G* level using Gaussian09.

Fig. 3  3D view of the docking interactions of the compound 1 (brown color, stick form at left side), compound 2 (dark blue color, stick form at right side), and Phosphoramidon (orange, ball and stick form in the middle) as standard with residues in the active site of Collagenase.

binding site in the main protease (Mpro, pdb: 6LU7 and 2GTB) models, requiring minimum energy of −5.83 kcal/mol and −6.32 kcal/mol, respectively (Table 2). The compound 1 exhibits the better activity than compound 2 against both main proteases. However, these small compounds have lower binding affinity than NL-1 and NL-2 as the standard compounds (−8.65 kcal/mol and −8.41 kcal/mol in Table 2).

In the other words, the docking results suggest that both compounds 1 and 2 tend to have lower efficacy on the main protease targets of SARS-CoV-2 than standard compounds, (NL-1 and NL-2). The reason for this is that the standard compounds interact strongly and more frequently with the target proteins, and the surface areas they cover on the active surface are much larger than the small ligand molecules studied. This situation is indicated visually in Figs. 5A and 5B, and numerically in Table 2.

3.3 Cancer results
The cytotoxic effect of test compounds on human pancreatic cancer cell lines is shown in Fig. 6. Compounds 6-hydroxy-4-methylcoumarin and 2,5-dihydroxyacetophe-
none significantly decreased human pancreatic cancer cell viability from low doses (Fig. 6). In addition, 100 µM dose of all compounds caused significant reductions in human pancreatic cancer cell viability (Fig. 6). In general, we can say that of the four tested compounds, 6-hydroxy-4-methylcoumarin and 2,5-dihydroxyacetophenone have cytotoxic effects in all cell types, and this effect is particularly strong in human pancreatic cancer cells.

4 Discussion

In this study, N-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone was used as a standard for elastase. For 2,5-dihydroxyacetophenone compound, a good result was determined compared to the standard, but a poor result was calculated for 6-hydroxy-4-methylcoumarin compound compared to the standard. Inhibiting the activity of extracellular matrix-degrading (ECM) proteins such as elastases and collagenases may be a useful approach to prevent UV-induced skin changes and premature skin...
aging. Because reactive oxygen species (ROS) plays a key role in the activation of these enzymes, scavenging ROS by natural antioxidant compounds may be an option for inhibiting such skin-degrading enzymes. Phenolic molecules are an important class of natural antioxidants. They belong to various subclasses of secondary plant metabolites classified as stilbenes, flavonoids, phenolic acids and lignans and are ubiquitous in the plant kingdom. Especially red and white grapes contain high amounts of phenolic acids and flavonoids such as catechin and gallic acid. Due to their chemical structure, polyphenols have strong antioxidant activities that scavenge a wide range of ROS such as superoxide radicals, hydroxyl radicals and Superoxide ($O_2^{-}$). In addition, polyphenols can inhibit the activity of proteolytic enzymes in vitro by acting as complexing or precipitating agents as noted in the literature. In particular, green tea polyphenols such as epigallocatechin gallate and catechin, which are widely used as ingredients in anti-aging skincare formulations, have been shown to exhibit moderate inhibitory effects against elastase and collagenase activity, possibly through non-covalent bonding.

Pancreatic cancer is known to spread rapidly and is rarely detected in early stages. Not until quite advanced stages, no signs and symptoms are observed and in advanced stages, it is nearly impossible to remove the tumor. This type of cancer is known to be aggressive and migrate to different sections of the body quickly. If metastasis of pancreatic cancer could be stopped it would be possible to manage the tumor without searching for additional tumor spread and if this treatment could be combined with drug resistance decrease it could be used as a good treatment option for pancreas cancer.

5 Conclusion
In this study, inhibition effects on both compounds, collagenase and elastase enzymes were determined, the results were obtained at micromolar level and micromolar acceptable. then the interactions between enzymes and compounds were examined with molecular docking and the results are similar and suitable to in vitro results, then Anti-COVID19 study was done with molecular docking program, for Anti-COVID the results are not as we wanted but not bad. We have studied the latest anticancer effects and obtained good results, so we can say that we can use these compounds in drug design in the future. The increase in elastase enzyme activity reveals the structural and functional changes of collagen and elastic fibers. This situation leads to the deterioration of the flexible structures of the skin, the formation of a rough texture and aging. Elastase inhibitors show anti-wrinkle activity that maintains skin elasticity. In addition, the imbalance between elastase and its natural inhibitors causes tissue damage and may cause lung and connective tissue diseases such as cystic fibrosis, asthma, pulmonary emphysema, respiratory distress syndrome. Therefore, it is thought that with the development of elastase-specific inhibitors, it will be possible to control elastase-related diseases.

Data Availability Statement
Data that support study findings are available with the corresponding author upon reasonable request.

Authors’ Contributions
All authors have had a same role in preparing, designing, doing experiments, analyzing, writing, and submitting the recent manuscript.

Conflict of Interest
There isn’t any conflict of Interest.
Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess22021

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