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Some phenolic compounds as inhibitors of glutathione S-transferase and glutathione reductase: an *in vitro* and *in silico* analysis

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Abstract: It has been determined that Glutathione S-transferases (GSTs) and Glutathione reductase (GR) activities increase in cancer cells and contribute to the progression of cancer by causing multidrug resistance (MDR). The enzyme glutathione reductase catalyzes the transfer of electrons between oxidized glutathione (GSSG) and nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH). The resulting reduced glutathione (GSH) can detoxify various xenobiotics by attacking the electrophilic center in the catalysis of GSTs. Detoxification of anticancer drugs with increased expression of GST and GR in cancer cells reduces the effectiveness of these drugs. GST and GR inhibition is an important approach in cancer treatments. In this study, from human erythrocytes, GR was isolated using 2',5'-ADP Sepharose 4B affinity chromatography method with 16.912 EU/mg protein specific activity, and GST was isolated by using the Glutathione Agarose affinity chromatography method with 4.88 EU/mg protein specific activity. After the isolation of the enzymes, the inhibition effects of vanillin, epicatechin, and catechin on the activities were investigated. None of the three substances inhibited GST. Vanillin and epicatechin were found to inhibit GR with IC_{50} values of 86.25 µM and 345 µM, respectively. The elucidation of the inhibition mechanism was carried out by molecular docking studies conducted using the AutoDock program. It has been thought that the findings obtained as a result of this study will guide the design of inhibitors in GR and GST targeted therapies.

Keywords: Glutathione, vanillin, epicatechin, catechin, inhibition, molecular docking.

Bazı fenolik bileşiklerle glutatyon s-transferaz ve glutatyon redüktaz inhibisyonu: *in vitro* ve *in siliko* analiz

Özet: Glutatyon S-transferazlar (GSTs) ve Glutatyon redüktaz (GR) aktivitelerinin kanser hücresinde arttığı ve çoklu ilaç direncine (MDR) sebep olarak kanserin ilerlemesine katkıda bulunduğu belirlenmiştir. Glutatyon redüktaz okside glutatyon (GSSG) ile indirgenmiş nikotinamid adenin dinükleotid fosfat (NADPH) arasında elektron transferini katalize eder. Bu reaksiyon sonucu oluşan redükte glutatyon (GSH) GSTs katalizliğinde çeşitli ksenobiyotiklerin elektrofilik merkezine saldırarak detoksifiye eder. Kanser hücrelerinde antikanser ilaçların artan GST ve GR ekspresyonu ile detoksifikasyonu bu ilaçların etkinliğini azaltır. Bu nedenle kanser tedavilerinde GST ve GR inhibisyonu önemli bir yaklaşımdır. Bu çalışmada, insan eritrositlerinden GR izolasyonu 2',5'-ADP Sepharose 4B afinite kromatografisi yöntemiyle 16,912 EÜ/mg protein spesifik aktiviteyle, GST izolasyonu ise Glutatyon Agaroz afinite kromatografisi yöntemiyle 4,88 EÜ/mg protein spesifik aktiviteyle gerçekleştirilmiştir. Enzimlerin izolasyonundan sonra vanilin, epikatekin ve katekinin aktiviteler üzerine inhibisyon etkisi incelenmiştir. Her üç madde de GST'yi inhibe etmezken, vanilin ve epikatekinin GR'yi sırasıyla 86.25 µM ve 345 µM IC_{50} değerleriyle inhibe ettiği belirlenmiştir. İnhibisyon mekanizmasının aydınlatılması ise AutoDock programı kullanılarak moleküler yerleştirme çalışmaları ile gerçekleştirilmiştir. Bu çalışma sonucunda elde edilen bulguların GR ve GST hedefli tedavilerde inhibitör tasarımına yön vereceği düşünülmektedir.

Anahtar Kelimeler: Glutatyon, vanilin, epikatekin, katekin, inhibisyon, moleküler yerleştirme.

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1. INTRODUCTION

The family of enzymes known as Glutathione S-transferases (GSTs) has a crucial function in protection against oxidative stress and detoxification the cellular environment. These enzymes catalyze the conjugation of glutathione with a wide range of electrophilic compounds, resulting in their neutralization and excretion from the body. GSTs are expressed in various tissues and cell types, with different subtypes showing specific associations with certain cellular functions and tissue types. For example, Due to its involvement in cancer development and drug resistance, GSTP1 has become an appealing target for the treatment of cancer [1,2].

The broad substrate specificity and detoxification function of GSTs make them essential in maintaining cellular homeostasis and protecting against environmental toxins and carcinogens. The expression of GSTs can be influenced by various factors, including genetic polymorphisms, environmental exposures, and disease states [3,4].

There is a close relationship between GST and glutathione reductase (GR), as GR plays a critical role in maintaining intracellular levels of reduced glutathione (GSH), which is essential for GST function. The regeneration of GSH from its oxidized form (GSSG), which is produced during some antioxidant enzyme-catalyzed processes, is carried out by GR [5,6].

GR is a ubiquitous enzyme which is essential for preserving the balance of redox inside cells by catalyzing the reduction of GSSG to its reduced form (GSH), which is essential for several cellular processes [6]. GR is highly expressed in various tissues, including liver, erythrocytes, and brain, where it plays a critical role in protecting cells against oxidative stress by regulating the levels of GSH. GR expression can be influenced by various factors, including genetic polymorphisms and environmental exposures, which can lead to altered cellular redox status and increased susceptibility to oxidative damage [6]. Given its central role in redox homeostasis, GR has emerged as a promising drug target for various diseases, including cancer, inflammatory diseases, and parasitic infections [7,8].

Given their involvement in drug metabolism and the detoxification of xenobiotics, GSTs have emerged as a promising drug target for various diseases, including cancer, inflammatory diseases, and parasitic infections. The development of GST inhibitors has the potential to improve treatment outcomes and overcome drug resistance [9,10]. This study will provide an overview of GSTs, their functions and expression, and their potential as a drug target for various diseases.
The development of GR inhibitors has the potential to improve treatment outcomes by disrupting redox balance in diseased cells and increasing their susceptibility to oxidative stress [6].

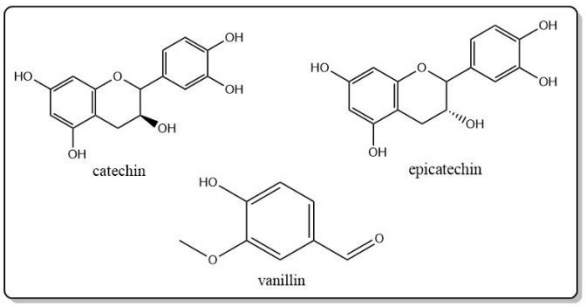

Figure 1. Structures of phenolic compounds that was used in inhibition studies

Phenolic compounds are a class of natural or synthetic compounds characterized by the presence of one or more phenol rings. These compounds exhibit diverse biological functions, such as antioxidative, anti-inflammatory, and antitumor effects. Due to their diverse pharmacological activities, phenolic compounds have become a focus of drug discovery and development [11].

So, in this study it was aimed to investigate the inhibitory effects of phenolics such as vanillin, epicatechin, and catechin on the activities of human erythrocytes GST and GR.

2. MATERIALS AND METHOD

2.1. Materials

Isolation of GR and GST enzymes was carried out using human blood obtained from healthy donors through the Turkish Red Crescent (Erzurum branch). The chemicals used in the experiments for isolating and testing activity were obtained from E. Merk AG, Fluka, and Benzoate derivatives from Fluorochem.

2.2. Activity assays

The activity of GST was determined according to the method proposed by Habig et al., (1974) with slight modifications [12,13]. Activity monitoring was carried out at 340 nm and 25ºC using a UV-Vis Spectrophotometer. The reaction medium consisted of 10 mM phosphate buffer (pH 6.5), 2 mM GSH, 2.5 mM CDNB, and enzyme eluate.

The GR activity was determined based on the method proposed by Carlberg and Mannervik, (1981) with slight modifications [14,15]. The activity measurement was conducted using a Beckman Coulter DU 730 UV/Vis Spectrophotometer at 340 nm. The assay medium consisted of 10 mM phosphate buffer (pH 7.5), 2 mM GSSG, enzyme eluate, and 0.2 mM NADPH.

2.3. Inhibition assays

To investigate effects of inhibition of the phenolic compounds on GST and GR enzymes, the proteins were first isolated from human blood using affinity chromatography techniques, as previously described in the literature. Hemolysate was prepared by separating erythrocytes from whole blood using the detailed procedure in Güller, (2021) [16].

GST and GR were isolated using Glutathione Agarose and 2′,5′-ADP Sepharose 4B affinity columns, respectively [13,15]. Three different phenolic compounds, namely catechin, epicatechin, and vanillin, listed in Table 1, were tested as inhibitors. Stock solutions of each inhibitor were prepared at a concentration of 1 mg/mL. To determine the IC_{50} values, enzyme activities were measured at varying concentrations of inhibitors.

2.4. Molecular Docking

The molecular docking study was conducted to investigate the possible types of interactions and energies between the molecules and the enzymes. AutoDock 4.2 was used for the study. The ligands were prepared by drawing the three-dimensional structures of the derivatives as *sdf files with ChemDraw software. The *sdf files were then converted to *pdb format using Avogadro software. The ligands were further prepared by creating rotatable torsions with the Autodock tool and saved as a *pdbqt file. The receptor protein used in this study was hGR (PDB code: 1XAN) [17] of which crystal structure (*pdb file type) were obtained from the protein database (http://www.rcsb.org/pdb). The AutoDock tool was used to prepare the proteins, and Kollman charges were added to them. The resulting proteins were saved in *pdbqt format [18,19].

3 RESULTS AND DISCUSSION

To investigate new inhibitors of GST and GR is important because these enzymes play critical roles in maintaining cellular homeostasis and protecting against oxidative stress and environmental toxins. However, their overexpression has been linked to various diseases, including cancer, inflammatory diseases, and parasitic infections. In particular, GSTs have been implicated in drug resistance, making them an attractive target for cancer treatment. Additionally, inhibition of GR activity can lead to increased levels of oxidized glutathione (GSSG), which has been associated with various diseases, including cancer and Alzheimer's disease [20,21]. Therefore, the development of new inhibitors of GST and GR has the potential to improve treatment outcomes for these diseases and overcome drug resistance [21,22]. One of the important applications of phenolic compounds in drug design is as inhibitors of target enzymes such as GST and GR. These enzymes play critical roles in cellular detoxification and protection against oxidative stress, but they are also involved in drug resistance in cancer and other diseases. Phenolic compounds have been shown to inhibit the activity of GST and GR, which can enhance the efficacy of chemotherapy drugs and overcome drug resistance. Furthermore, phenolic compounds are generally safe and well-tolerated, making them attractive candidates for drug development. Overall, the use of phenolic compounds as inhibitors of target enzymes in drug design offers a promising approach to developing new and effective treatments for a variety of diseases [23,24].

This study was focused on *in vitro* inhibition of human erythrocytes GR and GST by some phenolic compound and inhibition profiles were predicted by molecular docking analysis.

For *in vitro* inhibition studies, firstly, GR and GST were isolated from human erythrocytes with the specific activities of 16.912 EU/mg protein and 4.88 EU/mg protein respectively. Then, solutions of the compounds were prepared at a concentration of 1 mg/mL, and adequate dilutions were made until appropriate concentrations were reached. According to the inhibition studies, it was found that while catechin did not show an inhibitory effect, vanillin and epicatechin inhibited hGR with IC_{50} values of 86.25 µM and 345 µM, respectively (Figure 2).

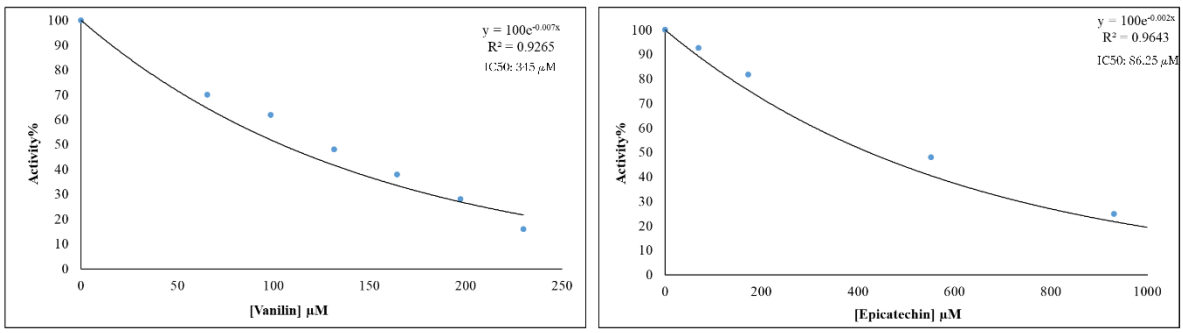

Figure 2. IC_{50} graphs of vanillin and epicatechin

Similar to this, the results of past studies have demonstrated that some natural substances inhibit GR isolated from various sources. In a study, β-sitosterol, stigmasterol, diosgenin, and jervine were found to have IC_{50} values of 1.2580 µM, 5.2116 µM, 0.1916 µM, and 0.7701 µM, respectively [25]. Another report revealed that the IC_{50} values of curcumin, quercetin, and resveratrol were 17.25 ± 3.8 mM, 57.8 ± 14.2 mM, and 520 ± 96.7 mM, respectively [26]. Güller, (2021) studied the effects of resorcinol and its derivatives on hGR, and found IC_{50} values to range from 0.014 to 1.500 mM [27]. As for GST inhibition, all three phenolic compounds did not inhibit the activity of hGST. In some studies, the effects of various phenolic compounds on the GST activity were investigated. Appiah-Opong et al., (2009) reported that curcumin and its analogues (diferuloymethane, bisdemethoxycurcumin, demetoxydiferuloymethane) showed an inhibitory effect against a number of GST isoenzymes [28]. van Zanden et al., (2003) in *in vitro* experiments, found that quercetin inhibited the hGSTP1-1 enzyme and the IC_{50} value was 4.4 µM [29].

In this study, the free binding energies of the inhibitors-GR receptor complex were determined using the AutoDock4 tool. The accuracy of the docking process was confirmed by the redocking of co-crystallized ligands and receptor proteins, which resulted in an RMSD value of 0.076, as depicted in Figure 3.

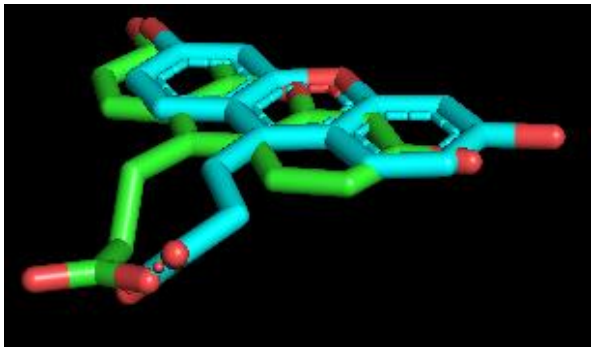

Figure 3. Result of re-docking process on hGR receptor

Following the validation process, docking experiments of ligands into receptor was conducted. According to the results presented in Table 1, all the compounds exhibited lower binding affinities compared to the co-crystallized ligand, HXP, which had a binding affinity of -8.11 kcal/mol.

Table 1. Inhibition results of compounds on hGR.

Compounds	IC_{50} values (µM)	Estimated Free Energy of Binding (kcal/mol)	Estimated Inhibition Constant, K_i (µM)
Catechin	-	-	-
Epicatechin	345	-7.00	7.37
Vanillin	86.25	-4.88	263.28
HXP*	-	-8.11	0.978

*HXP: 3,6-dihydroxy-xanthene-9-propionic acid, standard inhibitor of hGR.

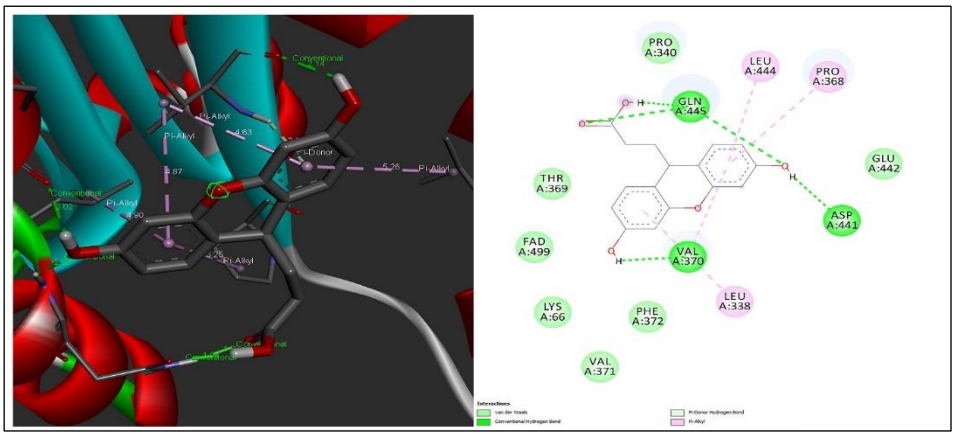

Figure 4. Docking pose of HXP, co-crystalized ligand, into GR receptor.

It was seen from Figure 4 that HXP interacted with Val370, Asp441, and Gln445 residues of receptor via six H-bond. It also showed hydrophobic π-alkyl interactions upon Leu338, Pro368, and Leu444 amino acids via π bonds of its benzene moieties.

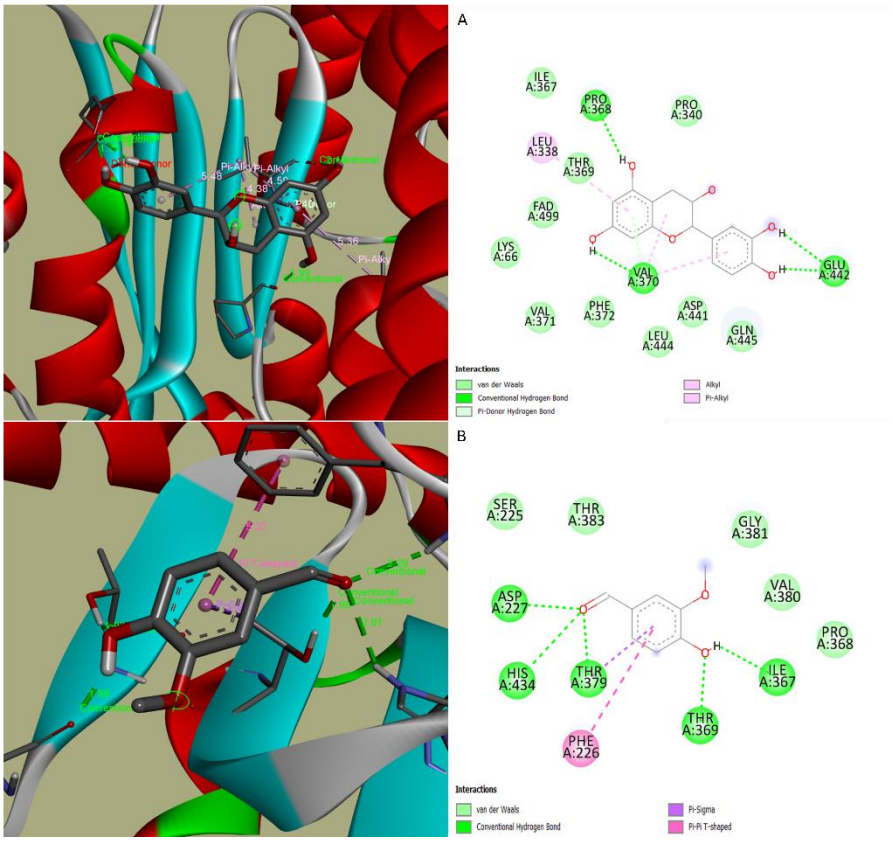

Figure 5. Docking poses of (A) epicatechin and (B) vanillin into GR receptor.

Epicatechin indicated its inhibition effect via four H-bonds. The benzene group of the compound was observed to interact with the active site residues through hydrophobic π-alkyl and alkyl interactions, leading to increased stability of the compound within the binding pocket. As for, vanillin, it had π-sigma and π- π Ti-shaped interactions with benzene moieties of Phe226 and Thr379 residues with its benzene ring. It has been thought that its inhibition potency could be attributed to five H-bonds indicated with binding pocket residues mostly being as H-acceptor.

4. Conclusion

In conclusion, this study provides an assessment of the inhibitory effects of catechin, epicatechin, and vanillin on human erythrocyte GR and GST, both *in vitro* and *in silico*. The inhibition of these enzymes has been recognized as a viable approach in the treatment of cancer. The results of *in vitro* inhibition study indicated that all three phenolic compounds did not inhibit activity of hGST. While catechin did not show inhibition effect, vanillin and epicatechin inhibited hGR with the IC_{50} values of 86.25 µM and 345 µM respectively. Free Binding energies of inhibitors on hGR were estimated as -4.88 kcal/mol and -7.00 kcal/mol. Their inhibitory potency was found lower than standard inhibitor, HXP.

Conflict of Interest

The author declares that there are not any known competing financial interests or personal relationships that could have appeared to influence the work reported in this article. This study was presented as an oral presentation at the "11th International Conference on Engineering & Natural Sciences, September 18-19, 2021 / Mus, Turkey".

Ethical Approval: Ethics Approval is not required for this study.

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