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Alteration of enzyme activities and kinetic properties of GST and NQO1 with naturally occurring phenolic compounds

GST ve NQO1 enzim aktivitelerinin ve kinetik özelliklerinin doğal olarak oluşan fenolik bileşikler ile değişimi

Abstract: Objective: Glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) are the enzymes important in cytoprotection and bioactivation of chemicals. This study has addressed effects of polyphenolic compounds; ellagic acid, quercetin, naringenin, resveratrol, rutin and hesperidin on rabbit liver GST and NQO1 enzyme activities.

Methods: Cytosolic fractions were obtained from homogenized liver tissues of rabbit by differential centrifugation. After calculating IC₅₀ values of individual enzymes for phenolic compounds, both Lineweaver-Burk and Dixon plots were drawn to determine the effect of phenolics on enzyme activity. Michaelis-Menten constant (Kₘ), maximum velocity (Vₘₐₓ), and inhibition constant (Kᵢ) were calculated from Lineweaver-Burk and Dixon plots, respectively.

Results: Resveratrol was found to be the most potent inhibitor for rabbit hepatic cytosolic GST activity with 75.9±2.06 μM IC₅₀ value while naringenin was the least potent one with IC₅₀ value of 260±1.92 μM. Hesperidin and quercetin were found to be the most and least potent inhibitors for NQO1 enzyme activity with IC₅₀ values of 2.7±0.85 μM and 13.8±0.91 μM, respectively. Resveratrol and naringenin inhibited GST activity noncompetitively and mixed type with Kᵢ of 6.2 μM and 245 μM, respectively; while both hesperidin with 0.64 μM Kᵢ value and quercetin with 3.5 μM Kᵢ value inhibited NQO1 activity in a competitive manner.

Conclusion: These results indicate that phenolic compounds may modulate Phase II enzyme, GST and NQO1. Moreover, they can influence the metabolic activation of xenobiotic and toxic compounds metabolized by this enzyme.

Keywords: GST, NQO1, polyphenols, inhibition kinetic, IC₅₀
bitor bulunmuştur. Hesperidin ve quersetin ise NQO1 enzimi için sırasıyla 2.7±0.85 μM IC_{50} değeri ile en fazla ve 13.8±0.91 μM IC_{50} değeri ile en düşük inhibitörler olarak bulunmuştur. Resveratrol ve naringenin GST aktivitesini sırasıyla 6.2 μM K_{i} değeriyle yansıtırlar ve 245 μM K_{i} değeriyle karşılaştırılır inhibisyon şeklinde inhibe etmiştir. Öte yandan 0.64 μM K_{i} değeri ile hesperidin ve 3.5 μM K_{i} değeri ile quersetin NQO1 enzimini yansıtırlar inhibisyon şeklinde inhibe etmiştir.

Sonuç: Bu bulgular göstermektedir ki fenolik bileşiklerin Faz II enzimlerinden GST ve NQO1’i modüle edebilir. Ayrıca, bu bileşikler bu enzimlerin metabolize ettiği ksenobiyotik ve toksik bileşiklerin metabolik aktivasyonunu etkileyebilir.

Anahtar Kelimeler: GST, NQO1, polifenoller, inhibisyon kinetiği, IC_{50}

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Introduction

The detoxification of xenobiotics is partly provided by the phase II enzymes, glutathione-S transferase (GST) and NAD(P)H quinone oxidoreductase 1 (NQO1). On the other hand, these enzymes catalyze the bioactivation of several compounds. Besides this, the inhibition of GST and NQO1 may be a strategy in cancer therapy in order to combat with the drug resistance of cancer cells to chemotherapeutics [1].

GSTs are involved in conjugation of reduced glutathione with several electrophilic compounds. Beside their positive actions, recent studies showed that some glutathione conjugates display toxic effects [2,3]. The toxic effect of these conjugates is resulted from the formation of unstable thiols and these compounds can be further converted to either alkylation agents or stable but toxic compounds such as dihaloalkanes containing dichloromethane and dibromoethane [4]. Moreover, some toxic glutathione conjugates are derived from hydroquinones and quinines by the action of GST resulting in a toxic effect [5]. In addition, GSTs have been shown to be over-expressed in tumor cells hence they increase the resistance to chemotherapeutic drugs. Cancer chemotherapeutic agents such as adriamycin, 1,3-bis (2-chloroethyl)-1 nitrosourea, busulfan and cyclophosphoamid are detoxified by GST [6]. Therefore, enhanced GST-mediated conjugations of cytostatics due to increased GST expression used in cancer treatment have been suggested among the mechanisms of drug resistance.

In this context, inhibition of GSTs may sensitize drug-resistant cells. Although, many attempts have been made to develop GST inhibitors many of those inhibitors such as synthetic analogues and glutathione conjugates are either too toxic to be used in vivo or are effective only in vitro. Furthermore, lacking stability and selectivity, and having a poor pharmacological profile, synthetic analogues cause problems when used [7].

NQO1, obligate two-electron reductase, has a protective role against natural and exogenous quinones. On the other hand, the product hydroquinones sometimes are not stable and NQO1 metabolism resulted with formation of more active product which autoxidizes to produce reactive oxygen species or undergo rearrangement to generate alkylationing species. Dinitropyrenes are found in diesel exhaust and the toxicity they cause is increased by the activation of NQO1 [8]. Moreover, activity of NQO1 has been found in elevated levels in some tumors such as in colon, liver and lung cancers [9,10]. The activation of some quinones by NQO1 can lead to formation of some compounds which can react with DNA by alkylationing the nucleophilic sites, which leads apoptosis [11,12]. NQO1 was also shown to have a significant role in mammalian cell cytotoxicity and genotoxicity of nitroaromatic pollutants, components of exhaust gases or residues of explosives in the environment as well as bioactivation of heterocyclic amines which are mutagenic and carcinogenic [13,14].

Regular intake of a variety of fruits and vegetables is a key factor that contributes to reduced risk of degenerative diseases and cancer. They contain a wide range of phenolic acids and flavonoids which are among the sources of natural antioxidants. Feeding of rats with quercetin was ~25% decreased GST and NQO1 enzyme activities [15].

This study was undertaken to elucidate the possible in vitro effects of certain plant phenolic compounds; ellagic acid, quercetin, naringenin, resveratrol, rutin and hesperidin as shown in Figure 1, for their ability to modulate rabbit hepatic GST and NQO1 enzyme activities. We hypothesized that these plant-derived polyphenols might exert some activity on these two key xenobiotic metabolizing enzymes. This paper explores and describes the nature of these interactions.

Materials and Methods

Chemicals

Ellagic acid, quercetin, rutin, hesperidin, naringenin, 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum
albumin (BSA), ε-aminocaproic acid (ε-ACA), phenylmethanesulfonyl fluoride (PMSF), glutathione reduced form (GSH), 2,6 dichlorophenol-indophenol (DCPIP) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) was taken from Applichem, Darmstadt, Germany. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

**Preparation of liver cytosols**

The procedures involving animals and their care were carried out in accordance with the Declaration of Helsinki and approved by the animal research ethics committee. Adult male New Zealand white rabbits, initially weighing 2.0–2.2 kg (three months old) were used in this study. Liver cytosols were prepared by differential centrifugation as described before [16–18]. Homogenization was carried out in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA and 0.1 mM PMSF by using a glass-teflon homogenizer and the liver homogenates were centrifuged at 10800 g (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) for 25 minutes at 4°C. The resultant supernatant was centrifuged at 145215 g (Sorval-Combi Ultracentrifuge Dupond Company, Newton, Connecticut, USA) for 45 minutes at 4°C. The supernatant obtained was kept at -80°C and used as a source of cytosol. The protein concentration of rabbit liver cytosols was determined by the method of Lowry et al. (1951) by using crystalline bovine serum albumin as a standard [19].

**Enzyme assays**

GST activities were determined spectrophotometrically by monitoring the thioether formation at 340 nm using CDNB as the substrate [20]. A typical assay mixture contained 41.6 mM potassium phosphate buffer pH 7.0, 1.3 mM GSH, and 1 mM CDNB in a final volume of 3 ml. Enzyme activity determinations were carried out at 25 °C and incubation mixtures without an enzyme source were also used as the blanks (non-enzymatic reactions). Enzyme activity was calculated by using an extinction coefficient of 9.6 mM⁴ cm⁻¹ [21].

Rabbit liver NQO1 enzyme activities were determined according to the method of Ernster (1967) as modified by Karakurt et al. (2013) based on the reduction of DCPIP inhibited by dicoumarol [22,23]. Standard enzyme assay mixtures contained 25 mM potassium phosphate buffer pH 7.8, 0.7 mg/ml BSA, 0.2 mM NADPH and 40 μM DCPIP in a final volume of 1 ml. DCPIP reduction reaction was followed continuously at 600 nm for 2 minutes by Schimadzu UV-160A UV visible spectrophotometer. The enzyme activity was calculated by using an extinction coefficient of 0.021 μM⁻¹ cm⁻¹.

Phenolic compounds, ellagic acid, quercetin, rutin, hesperidin, naringenin and resveratrol were dissolved in dH₂O or DMSO and prepared in different concentration and then used in enzyme reaction in the range of 0.05 µM to 500 µM. The final concentration of DMSO in the enzyme assays was held 2.5% and same amount of DMSO was also used in control groups. Concentrations of phenolic compounds used in the current work were chosen according to preliminary results obtained in our laboratory [18]. After calculating IC₅₀ values of individual enzymes for phenolic compounds, both Lineweaver-Burk and Dixon plots were drawn to determine the effect of phenolics on enzyme activity. Michaelis-Menten constant (Kₘ), maximum velocity (V_max), and inhibition constant (K) were calculated from Lineweaver-Burk and Dixon plots, respectively.

**Statistical analysis**

Statistical analyses were performed by PAIRED samples t-test using SPSS statistical software package for Windows.
All results were expressed as means with their Standard Error of Means (SEM). The data were analyzed and the kinetic constants were calculated using the following equations:

Michaelis Menten equation: \( V = \frac{V_{max} * [S]}{K_m + [S]} \)  \( (1) \)

Noncompetitive inhibition: \( V = \frac{V_{max} * [S]}{(K_m + [S]) * \alpha'} \)  \( (2) \)

Competitive inhibition: \( V = \frac{V_{max} * [S]}{(K_m * \alpha' + [S])} \)  \( (3) \)

\( \alpha' = (1 + [I] / K_i) \)

In this equation, \( V \) is the reaction rate, \([S]\) is the substrate concentration, \( V_{max} \) is the maximal velocity, \([I]\) is the inhibitor concentration, \( K_m \) is the Michaelis-Menten constant and \( K_i \) is the inhibition constant.

### Results and Discussion

GST and NQO1 enzymes have important functions in the metabolism and detoxification of a large number of xenobiotics [6,24]. Besides, these enzymes have been involved in the production of more reactive or more toxic products. Such as conjugation of parent compounds, vicinal dihalogenoalkanes and hexachlorobutadiene resulted in more reactive species [25]. Immunoprecipitation of NQO1 was demonstrated that human and rabbit NQO1 has very similar polypeptide composition [26]. Inhibition of GST and NQO1 enzymes are not only important to prevent conversion of procarcinogens to carcinogens, but also important for endogenous metabolism. Regulation of the biosynthesis of a number of important arachidonic acid metabolites such as prostaglandins and leukotrienes was demonstrated that they are controlled by GST and NQO1 enzymes [27,28]. Furthermore, inhibition of GST and NQO1 may be a strategy in cancer therapy in order to combat the drug resistance of cancer cells against chemotherapeutics [29]. Antioxidant, anti-inflammatory, and antiviral activities of phenolic compounds have been demonstrated to be preventive in coronary heart disease, stroke, and certain cancers [30,31]. The objective of the present study was to evaluate in vitro effect of polyphenolic compounds; ellagic acid, quercetin, naringenin, resveratrol, rutin and

**Table 1**: Inhibitory effect of polyphenolic compounds on rabbit liver GST and NQO1 activities. \( IC_{50} \) values are given as \( \mu \)M. Concentrations of compounds are their final reaction concentrations. All values are expressed as means±SE for two different experiments.

<table>
<thead>
<tr>
<th>Polyphenolic compounds</th>
<th>( IC_{50} ) (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GST</td>
</tr>
<tr>
<td>Ellagic Acid</td>
<td>142±6.36</td>
</tr>
<tr>
<td>Quercetin</td>
<td>80±1.38</td>
</tr>
<tr>
<td>Naringenin</td>
<td>260±1.92</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>75.9±2.06</td>
</tr>
<tr>
<td>Rutin</td>
<td>164±3.42</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>242±4.17</td>
</tr>
</tbody>
</table>

**Table 2**: Kinetic parameters, \( K_m, V_{max} \) and \( K_i \) were determined from the Lineweaver-Burk and Dixon plots. Values represent the means±S.E.M. of duplicate determinations.

<table>
<thead>
<tr>
<th>Phenolics compounds</th>
<th>Enzymes</th>
<th>( K_m ) (( \mu )M)</th>
<th>( V_{max} ) (( \mu )mol/min/mg)</th>
<th>( K_i ) (( \mu )M)</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td>GST</td>
<td>0.9</td>
<td>322–212</td>
<td>65</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.005–0.023</td>
<td>57.3</td>
<td>0.6</td>
<td>Competitive</td>
</tr>
<tr>
<td>Quercetin</td>
<td>GST</td>
<td>0.64–1.03</td>
<td>272</td>
<td>61.5</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.024–0.056</td>
<td>78.9</td>
<td>3.5</td>
<td>Competitive</td>
</tr>
<tr>
<td>Naringenin</td>
<td>GST</td>
<td>2.5</td>
<td>7.3–6.8</td>
<td>314</td>
<td>Mixed Type</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.07–0.04</td>
<td>120–54</td>
<td>2.3–4.2</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>GST</td>
<td>0.4</td>
<td>7.45–6.31</td>
<td>6.2</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.04–0.02</td>
<td>93–45</td>
<td>4.4–3.2</td>
<td>Uncompetitive</td>
</tr>
<tr>
<td>Rutin</td>
<td>GST</td>
<td>0.9</td>
<td>5.6–5.1</td>
<td>245</td>
<td>Mixed Type</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.15</td>
<td>222–131</td>
<td>1.7</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>GST</td>
<td>0.437–0.781</td>
<td>0.142</td>
<td>143</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td>NQO1</td>
<td>0.045–0.078</td>
<td>87.5</td>
<td>0.64</td>
<td>Competitive</td>
</tr>
</tbody>
</table>
hesperidin on the rabbit liver cytosolic GST and NQO1 enzymes. For that purpose, rabbit liver cytosolic fractions were incubated with various concentrations of those phenolic compounds in the enzyme assay system. Polyphenolic compounds showed a distinct inhibitory effect on rabbit liver GST and NQO1 enzymes in a concentration dependent manner.

Ellagic acid inhibited hepatic GST and NQO1 activities in a dose-dependent manner with IC\textsubscript{50} values of 142±6.36 µM and 4.4±0.37 µM, respectively (Table 1). At 250 µM, ellagic acid completely inhibited the rabbit hepatic GST activity, while 30 µM of ellagic acid was required for complete (100%) inhibition of NQO1 activity. Kinetic parameters of GST and NQO1 in the presence for polyphenolics determined from the Lineweaver-Burk and Dixon plots were given in Table 2. The Lineweaver-Burk plot (1/V versus 1/[S]) given in Figure 2 indicated that the K\textsubscript{m} remained unchanged by the presence of different concentrations of ellagic acid for GST enzyme, while V\textsubscript{max} decreased with an increase in ellagic acid concentration. The apparent K\textsubscript{m} value of GST was found to be 0.9 mM for CDNB in all concentrations of ellagic acid present in the reaction medium. V\textsubscript{max} values of the GST enzyme reaction were decreased from 212 µmol/ml/mg in the presence of 30 µM ellagic acid in the reaction medium. On the other hand, for NQO1 enzyme, an increased K\textsubscript{m} and a constant V\textsubscript{max} were observed. K\textsubscript{m} value was increased from 5 µM to 23 µM with increasing ellagic acid concentration. For NQO1 enzyme, in all concentration of ellagic acid, V\textsubscript{max} remain unchanged as 57.3 µmol/ml/mg. Dixon plot, 1/V versus 1/[Inhibitor], plotted in the presence of different fixed concentrations of CDNB ranging from 0.2 mM to 1 mM and DCPIP ranging from 25 µM to 45 µM, used to determine the type of inhibition and to calculate the K\textsubscript{i} value. From the intersecting point of the four lines, ellagic acid was found to be a noncompetitive inhibitor for GST and competitive inhibitor for NQO1 enzyme with K\textsubscript{i} of 65 µM and 0.6 µM, respectively.

Resveratrol has protective effect against cardiovascular diseases [32]. Resveratrol plays an important role in the inhibition of tumor initiation, promotion and progression [33]. The inhibitory effects of resveratrol have also demonstrated on human breast cancer cells and prostate cancer [34,35]. In the present study, as shown in Table 1, resveratrol compared to naringenin was found to be 3.46 fold more potent inhibitor for rabbit liver GST. The strength of GST inhibition by polyphenols used in this study was observed in the following order, resveratrol>quercetin>ellagic acid>rutin>hesperidin>naringenin. IC\textsubscript{50} values of quercetin, naringenin, resveratrol, rutin and hesperidin on GST enzyme activity were also determined and given in Table 1 as 80±1.38 µM, 260±1.92 µM, 75.9±2.06 µM, 164±3.42 µM, and 242±4.17 µM, respectively. K\textsubscript{m} constants of quercetin, naringenin, resveratrol, rutin and hesperidin for GST enzyme obtained from Dixon plot were found as 61.5 µM (competitive), 314 µM (mixed type), 6.2 µM (noncompetitive), 245 µM (mixed type) and 143 µM (competitive), respectively as indicated in Table 2. The inhibition types of GST enzyme by each polyphenol were also written in parenthesis and given in the same Table.

Like ellagic acid; quercetin, naringenin, resveratrol, rutin and hesperidin also inhibited rabbit liver NQO1 activity dose-dependently. Hesperidin compared to quercetin was shown as 5.11 fold more potent inhibitor for NQO1 enzyme and the strength NQO1 inhibition by polyphenols used in this study was observed in the following order, hesperidin=naringenin>ellagic acid>resveratrol>rutin>qu
er cetin. IC\textsubscript{50} values of quercetin, naringenin, resveratrol, rutin and hesperidin on rabbit liver NQO1 enzyme activity were determined as 13.8±0.91 µM, 2.7±0.33 µM, 4.9±1.35 µM, 13±0.71 µM, and 2.7±0.85 µM, respectively (Table 1). K\textsubscript{i} constants of quercetin, naringenin, resveratrol, rutin and hesperidin for NQO1 enzyme obtained from Dixon plot were found as 3.5 µM (competitive), 2.3 µM-4.2 µM (uncompetitive), 4.4 µM-3.2 µM (uncompetitive), 1.7 µM (noncompetitive) and 0.64 µM (competitive), respectively (Table 2). As a representative figure for kinetic studies of polyphenolic compounds, ellagic acid for GST and hesperidin for NQO1 were given in Figure 2 and 3, respectively. However, a complete picture of effects of all polyphenols used in the present study on GST and NQO1 enzymes were given in Table 2. As competitive inhibitors, quercetin and hesperidin reduce the concentration of free enzyme available for substrate binding since they bind active site of the enzyme and temporarily block binding of substrate. On the other hand, uncompetitive inhibitors, resveratrol and naringenin, binds directly to the enzyme-substrate complex and are envisioned to cause structural distortion of the active site, thereby rendering the enzyme catalytically inactive. Mixed or noncompetitive inhibitors such as rutin can bind both the enzyme and enzyme-substrate complex. Calculated IC\textsubscript{50} values using log [Concentration of phenolic compound] vs percentage of inhibition plot shows that inhibition potential of each phenolic compounds has species selectivity. Therefore action of phenolic compounds has distant effects on rat, rabbit and human GST and NQO1 enzyme activities.

After consumption of 2 g of resveratrol, blood concentration of resveratrol and hesperidin were found as 5.5 µM and 1.28 µM at stead state [36,37]. Therefore, regular consumption of those flavonoids may affects GST and NQO1 enzyme activities in view of K\textsubscript{i} values 4.4 µM and 0.64 µM, respectively.

In conclusion, the present study demonstrated that plant phenolic compounds have different effects and mainly they are the inhibitors of GST and NQO1 enzymes in rabbit liver. Resveratrol and hesperidin inhibited GST and NQO1 enzyme activities to a great extent, respectively. Since these enzymes play important roles in the bioactivation of several carcinogens, it is likely that those phenolic compounds may modulate metabolism of several carcinogens by inhibiting GST and NQO1.

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Conflict of Interest: The authors have no conflict of interest.

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