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Original Article

Inhibitory Effects of Some Flavonoids on Thioredoxin Reductase Purified from Chicken Liver

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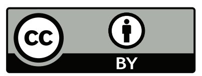
■Keywords

Flavonoids; inhibitors; natural products; thioredoxin reductase.



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ABSTRACT

Thioredoxin reductases (TrxRs) are selenocysteine-containing flavoenzymes that reduce Trxin NADPH-dependent manner. In the view of the direct vital role of TrxR in a wide range of biochemical and physiological processes, methods to inhibit this enzyme are clinically important. TrxR has recently emerged as a new candidate in anticancer drug investigations because of overexpression in tumorous cells. In this study, TrxR from chick liver was purified 94.6-fold with a yield of 4.86% and a specific activity of 0.19 EU/mg. K_M and V_{max} values of TrxR for DTNB were calculated as 0.9 mM and 0,03 EU/mL, respectively. Then, the effects of the flavonoids hesperidin, naringenin, chlorogenic acid, ferulic acid, naringin, 3,4-dihydoxybenzoic acid, and ellagic acid on the enzyme activity were evaluated under *in-vitro* conditions. Ellagic acid showed the strongest inhibitory activity on TrxR with a IC_{50} value of 18 µM, followed by naringenin and chlorogenic acid with IC_{50} values of 46.7 µM and 75.8 µM, respectively. Our results showed that flavonoids as natural products are potential inhibitors of TrxRs and the flavonoid content in animal diets may alter metabolic pathways by influencing TrxR activity.

INTRODUCTION

All organisms possess effective enzyme systems in their metabolism (Hirt *et al*., 2002; Arnér& Holmgren, 2000). The thioredoxin system plays a vital role in cell functions by regulating cell redox signaling and redox environment (Montano *et al*., 2014), and consists of thioredoxin (Trx), NADPH, and thioredoxin reductase (Holmgren & Lu, 2010). Thioredoxin reductases (EC 1.8.1.9) are selenium-containing flavoenzymes that reduce NADPH into Trx (Akyol &Kuzu, 2017; Arner, 2009). Two known types of TrxR are present in the living world. The first one, a homodimer with 35 kDa subunits, is found in prokaryotes, yeast and plants, the second, a homodimeric structure with 55-65 kDa subunits, is found in some lower eukaryotes and animals (Pacitti *et al*., 2014). Two major TrxR isoforms are located in different cellular microenvironments inside the cell. TrxR1 is cytoplasmic protein, also found in the nucleus, whereas TrxR2 is expressed in mitochondria. Although the two isoforms have different gene expression patterns and are located different cell compartments cell, their structures and catalytic mechanisms show same features (Duan *et al*., 2014).

TrxR is an essential enzyme found in all living creatures and its level in tumorous cells is 10-fold or higher than that present in normal cells (Becker *et al*., 2000). Due to its overexpression in tumorous cells, TrxR has attracted the attention of researchers as a possible candidate for anticancer therapies (Lu *et al*., 2006; Choi *et al*., 2002; Soini *et al*., 2001). TrxR has been regarded as a key therapeutic target for the design of novel anticancer drugs and inhibitors of the thioredoxin system, and

play promising role in anticancer treatments (Zhou *et al*., 2013). To date, an increasing number of TrxR inhibitors have been identified, including ions (Temel *et al*., 2017), nitrosureas, organochalcogenides, texaphyrins (Bindoli *et al*., 2009), gold- and platinum-containing compounds (Cai *et al*., 2012), nitroaromatic compounds (Urig & Becker, 2006), drugs (Wang *et al*., 2008), biometabolites (Powis *et al*., 2006), which are potential TrxR inhibitors with a nanomolar to micromolar IC_{50} range.

Natural product derivatives are valuable candidates for the treasure of pharma and have played a crucial role in the discovery of novel therapeutics since the ancient times of human medicine. They bind to specific protein-based targets and have influence on their biochemical/physiological functions. Therefore, discovery of new therapeutics and their biomolecular targets have been important in the past decades (Duan *et al*., 2014). Flavonoids have been one of the important groups of plant-based compounds which show significant activity in several defects and diseases (Aksoy-Sagirli *et al*., 2015; Hettihewa *et al*., 2018). A significant number of studies on potential anticancer activity of flavonoidshas been carried out (Chahar *et al*., 2011; Batra & Sharma, 2013; Martinez-Perez *et al*., 2014; López-Lázaro, 2002). Considering these two valuable factors, i.e.,TrxR and flavonoids, we decided to investigate the biological activity of flavonoids on TrxR.

In this study, some common flavonoids, which are known to have several biological activities and a represent in poultry feedstuffs were determined. The aim of this study is to investigate the inhibitory effects of some common flavonoids on the enzyme thioredoxin reductase purified from chicken liver.

MATERIAL AND METHODS

Chemicals

Sodium dodecyl sulfate (SDS), sodium chloride, sodium acetate, hydrochloric acid, phosphoric acid, ethanol, methanol, isopropanol, acetic acid, sodium acetate, potassium chloride, ethylenediamine tetraacetic acid (EDTA), and β-mercaptoethanol were purchased from Merck (Darmstadt, Germany); hesperidin, naringenin, chlorogenic acid, ferulic acid, naringin, 3,4-dihydoxybenzoic acid, and ellagic acid were obtained from Sigma (Munich, Germany)and other chemicals required for the investigation steps were acquired from Sigma-Aldrich (St. Louis, MO, USA) and used without any additional purification. Deionized water from the UV version of Millipore, Direct-Q 3, was used for the preparation of the solutions in the investigations.

Natural source

Chick liver was selected as natural source for thioredoxin reductase. The livers were purchased from a poultry farm in Ağrı, Turkey, and brought to the laboratory in accordance with the cold chain procedures.

Homogenate preparation and heat denaturation

A buffer solution of 10 mM Tris/HCl (pH 7.5) with 1 mM ethylenediamine tetraacetic acid (EDTA) was used to wash the liver tissues, and the same buffer was used to prepare liver lysate with the aid of a homogenizer. The lysate was then centrifuged at the rate of 12,700 rpm for 30 min. After this process, the precipitate was firstly discarded and then the supernatant was removed. This supernatant was stored at 60°C for 9-10 min and then centrifuged for the second time at the rate of 12,700 rpm for 30 min to remove the precipitate and the supernatant. Filter paper was utilized to filtrate the supernatant, which was then applied to the affinity column.

Purification with affinity chromatography

A column with 10 mL bed volume was designed with 3 g dried 2’,5’-ADP Sepharose 4B. Distilled water (300 mL) was flushed through the gel system to remove any undesired particles and air. The system was then suspended in 10 mM Tris/HCl buffer with 1 mM EDTA (pH 7.5) and packed into the column. After the gel precipitation step, the chromatographic column system was equilibrated with the same buffer using a peristatic pump at the flow rate of 30 mL/h for the washing and equilibration steps. For the purification of target enzyme (TrxR), the biosolution was injected into the 2’,5’-ADP Sepharose 4B column system and the affinity column was washed with the equilibration buffer and 0.15 M K-phosphate buffer with 2 mM EDTA (pH 7.5) until final absorbance difference fixed at 0.05 at 280 nm. $NADP^+$ equilibration buffer with a 0 to 10 mM gradient was used to elute TrxR and the active biomolecules were obtained and dialyzed with equilibration buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed after the TrxRpurification step. TrxR purity grade and monomer molecular

mass were obtained in SDS-PAGE with 3% and 10% acrylamide for stacking and running gel, respectively, with 0.1 SDS according to the procedure of Laemmli (Laemmli, 1970).

Protein determination

Protein content during homogenate preparation and purification steps was spectrophotometrically determined at 595 nm, according to the Bradford method with the use of bovine serum albumin as standard (Bradford, 1976).

Enzyme activity determination

TrxR activity was spectrophotometrically assayed at 412 nm absorbance using a Shimadzu spectrophotometer (UV-1800). The assay is based on the NADPH-dependent reduction of the substrate, reacting with 5,5 -dithio-bis(2-nitrobenzoic acid) (DTNB). The enzymatic reaction was carried out in a total volume of 1 mL, 200 µM NADPH, 2 mM DTNB and 1 mM EDTA in 300 mM K-phosphate buffer (pH 7.75). A value of 13.6 mM^{-1} cm^{-1} for the extinction coefficient of DTNB was applied for the calculations.

Kinetic studies and *in-vitro* inhibition assay

To obtain the DTNB K_M values of the TrxR enzyme, enzymatic measurements were assayed using five different DTNB concentrations. Lineweaver-Burk graphs were drawn from the obtained data and the K_M value was calculated from these graphs (Lineweaver & Burk, 1934). To determine the effects of flavonoids on the TrxR enzyme, enzyme activity was measured using saturated substrate concentration and five different flavonoid concentrations as follows: hesperidin (0.0066-0.33 mM), naringenin (0.011-0.11 mM), chlorogenic acid (0.00564-0.282 mM), ferulic acid (0.155-1.55 mM), naringin (0.052-0.78 mM), 3,4-dihydoxybenzoic acid (0.13-1.3 mM) and ellagic acid (0.001-0.02 mM). The flavonoid concentrations that caused 50% inhibition (IC50) were obtained via the graphs of % activity-flavonoid concentration. Cheng-Prusoff equation was used to calculate Ki constants (Yung-Chi & Prusoff, 1973).

RESULTS AND DISCUSSION

Purification of TrxR from natural source

After lysis and clarification, purification procedure was carried out. TrxR purification was performed in two steps with the use of heat denaturation and chromatography step including affinity matrix of 2’,5’-ADP Sepharose 4B. The affinity matrix is used to purify the enzymes which use $NADP^+$ and NADPH coenzymes because of their strong affinity features against these kinds of enzymes (Adem & Ciftci, 2012). As a result of the purification step, TrxR from chick liver was purified 94.6-fold with a yield of 4.86% and a specific activity of 0.19 EU/mg, as shown in Table 1.

Table 1 – Affinity purification procedure for TrxR from chick liver.

Fractions	Activity (EU/mL)	Volume (mL)	Protein (mg/mL)	Total activity (EU)	Specific activity (EU/mg protein)	Recovery 100%	Purification fold
Homogenate	0.081	24	39.76	1.944	0.0020	100	1
Heat denaturation	0.074	19	18	1.406	0.0041	72.33	2.0
2’,5’-ADP Sepharose 4B	0.021	4.5	0.109	0.0945	0.1927	4.86	94.6

Lineweaver-Burk plots were drawn by measuring five different DTNB concentrations for the calculation of K_M and Vmax values. K_M and V_{max} values of TrxR for DTNB were calculated as 0.9 mM and 0.03 EU/mL, respectively.

SDS-PAGE analysis

To verify the purified enzyme from affinity chromatographic column was obtained with SDS-PAGE analysis and one single band was plotted from the gel (Figure 1). As shown in Figure 1, the bands obtained with the purification procedure were clear, consistent, showing good integrity and no diffusion. The mobility of units and/or subunits in SDS-PAGE indicates their molecular weight (MW) (Wang *et al.*, 2016), andMWsof SDS-PAGE bands are calculated according to standard biomarkers (Guo *et al.*, 2017). The MW of the purified enzyme was calculated as ~54 kDa with the use of Rf-log MW as standard biomarkers. β-galactosidase (116 kDa), lactorferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) were used as standards in Line 1.

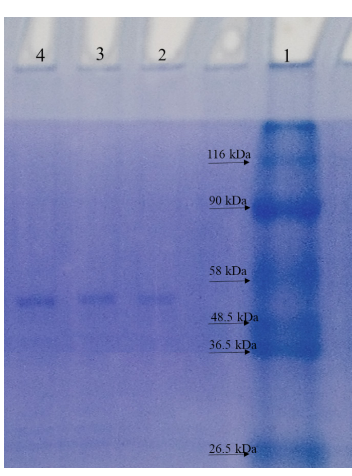


Figure 1 – SDS-PAGE image of the enzyme purified from chick liver.

Other studies indicate that TrxR from rat liver has a molecular weight of 58 kDa (Luthman & Holmgren, 1982), 56 kDa as mitochondrial enzyme from bovine adrenal cortex (Watabe *et al*., 1999), 55.2 kDa from human placenta (Gromer *et al*., 1998), 70 kDa from trout liver and 64.1 kDa from trout gill tissues (Akyol & Kuzu, 2017).

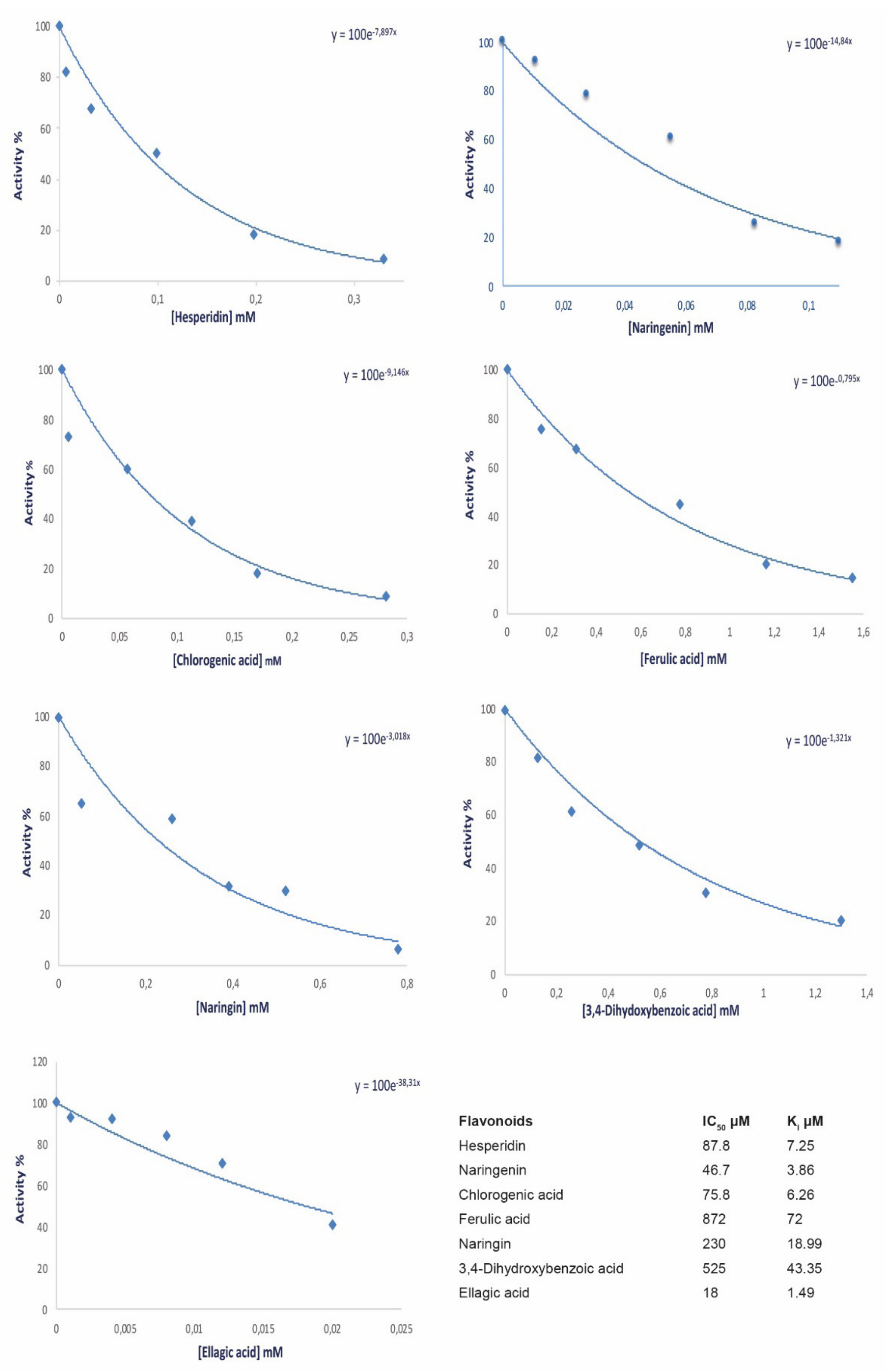
Effects of flavonoids on the enzymatic activity

Understanding the physiological and biochemical mechanism of the cellular biotargets is a vital mission for scientists (Zhang *et al*., 2016). Numerous antitumor drugs/ligands exert their biological effects through biometabolic pathways of enzyme inhibition. Therefore, enzyme inhibition studies have become increasing important in pharmaceutical research. TrxR is one of the biomolecular targets containing a selenocysteine on the flexible C-terminal arm, which is easily accessible to ligands during catalysis. Numerous electrophilic ligands may irreversibly and selectively interact with this active amino acid site (Urig & Becker, 2006). In this context, we hypothesized that TrxR inhibition may be an essential point causing the inhibition of tumor growth by blocking redox-dependent biological effects of thioredoxin.

In recent years, many research groups have worked on ligands that affect enzyme activity. It was demonstrated that some compounds are able to inhibit TrxR enzymatic reaction rate by 50%or more (Cortés *et al*., 2001; Deponte *et al*., 2005). Here we would like to give a brief overview of natural TrxR inhibitors in the literature.

Though this study has emphasized the significance of TrxR inhibition from the perspective of its antitumor activity, TrxR plays a major physiological role in several defects and diseases, such as AIDS, rheumatoid arthritis, reperfusion injury, etc. Therefore, TrxR inhibitors have several pharmaceutical applications in these defects and diseases as well as cancer. The inhibitory effect of some heavy metal ions, including Ni^{2+}, Cu^{2+}, Pb^{2+}, Cr^{3+}, Fe^{3+}, and Ag^{+}, on TrxR purified from rainbow troutgill tissues (Akyol & Kuzu, 2017) was evaluated, and Ag^{+} showed the strongest inhibitory effect on mitochondrial TrxR. The active site of TrxR contains the amino acid cysteine (Cys), and it is reported that the active sites and/or catalytic regions of enzymes containing Cys are inhibited by metal ions such as Hg^{2+} and Ag^{+} (Akyol & Kuzu, 2017). Curcumin is a major polyphenol molecule derived from *Curcuma longa*. The biomolecule has been therapeutically used in traditional medicine over the centuries, and has shown several therapeutic effects on cellular mechanisms of different diseases such as cancer (Teiten *et al*., 2010). Fang et al. (2005) showed that curcumin, which is used daily by millions of people, inhibits TrxR activity in rats at a IC_{50} value of 3.6 mM after incubation at 25°C for 2 h *in vitro*. Quinones are natural compounds used in several applications in life and medical sciences. They act as electron carriers in photosynthesis and they are used to treat some diseases and defects due to their antioxidant and therapeutic characteristics (El-Najjar *et al*., 2011). Xu & Arnér (2012) have demonstrated the inhibitory effects of pyrroloquinoline quinones (PQQs) on TrxR. PQQs are active redox cofactors of bacterial quinoproteins and, in micromolar concentrations, effectively inhibit the activity of TrxR. Isothiocyanates occur in a wide range of vegetables and have numerous biological and pharmacological properties. These natural compounds are powerful inhibitors for carcinogenesis (London *et al*., 2000). According to Jakubíková and colleagues (2006), isothiocyanates inhibit the activity of cellular TrxRbyTrx accumulation. Flavonoids are compounds derived from vegetables and fruits, and it is claimed they have several health promoting actions due their biochemical and pharmacological characteristics (Lautraite *et al*., 2002). Several research groups have investigated the potential of flavonoids to inhibit carcinogenesis (Liu *et al*., 2008; Zhang *et al*., 2004). Lu et al. (2006) examined the bioactivities of some flavonoids on TrxR and found that myricetin (IC_{50} value of 0.62 mM) and quercetin (IC_{50} value of 0.97 mM) have strong inhibitory characteristics on mammalian TrxR. Powis et al. (2006) evaluated the inhibitory activity analogs of a fungal metabolite, palmarumycin, on human TrxR and MCF-7 breast cancer cells, and reported that PX-960 showed the strongest inhibitory effect (IC_{50}: 0.27 μM) against human TrxR, where as palmarumycin CP1 was the most effective against MCF-7 breast cancer cells, with an IC_{50} value of 1.0 μM.

To the best of our knowledge, there are no experimental data on the bioactivities of flavonoids on chick liver TrxR. In the present study, chicken liver TrxR was treated with various concentrations of commercial flavonoids, including hesperidin, naringenin, chlorogenic acid, ferulic acid, naringin, 3,4-dihydoxybenzoic acid, and ellagic acid, to evaluate their bioactivity against TrxR. As illustrated in Figure 2, chicken liver TrxR activity was inhibited by the evaluated flavonoids in a dose-dependent manner. Ellagic acid showed the strongest inhibitory effect on TrxR, followed by naringenin and chlorogenic acid.



Flavonoids	IC_{50} µM	K_i µM
Hesperidin	87.8	7.25
Naringenin	46.7	3.86
Chlorogenic acid	75.8	6.26
Ferulic acid	872	72
Naringin	230	18.99
3,4-Dihydroxybenzoic acid	525	43.35
Ellagic acid	18	1.49

Figure 2 – % Activity-[I] plots of the tested flavonoids.

CONCLUSIONS

The cytosolic TrxR enzyme was first purified from chicken livers by electrophoretic homogenization, and the molecular weight of the enzyme was calculated. Briefly, we have demonstrated that flavonoids, as natural products, are potent inhibitors of thioredoxin reductase activity. This finding indicates that the content of flavonoids in animal diets may alter metabolic pathways by influencing TrxR activity. In addition, the

synthesis of flavonoids and their derivatives is pharmacologically important in further studies targeting the inhibition ofTrxR activity.

AUTHOR CONTRIBUTIONS

The authors conceived, designed the experiments and contributed equally to this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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