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Comparison of natural NQO2 inhibitors as a new target for cancer treatment in different cell lines

Kanser için yeni bir tedavi hedefi olan NQO2'nin doğal inhibitörlerinin farklı hücre hatlarında karşılaştırılması

Abstract: Objective: Quinones are highly reactive compounds undergoing either one- or two-electron reductions. Enzymatic one-electron reduction generates unstable semiquinone radicals which readily undergo redox cycling over NQO1 and NQO2 enzymes, in the presence of molecular oxygen, leading to the production of highly reactive oxygen species (ROS). NQO1 is responsible for detoxification where NQO2 inhibitors are found to show potent anticancerogenic effects. The aim of this study is to investigate some natural inhibitors of the NQO2 enzyme and to identify their action mechanisms over this enzyme on different cancer cell lines.

Methods: First of all the NQO2 inhibitory effects of flavonoids were investigated over enzyme kinetics and K_i values were calculated. Then the cytotoxic effects of flavonoids on different cancer cell lines such as Jurkat Clone E-6, MCF7 and HepG2 cancer cell lines were investigated via MTT test and their effects on NQO2 protein expressions via western blotting.

Results: K_i values for NQO2 inhibitory effects of the studied flavonoids are respectively, Apigenin (0.25 nM) < Kaempferol (0.55 nM) < Quercetin (1.06 nM) < Luteolin (1.54 nM). The IC_{50} values for flavonoids in three different cell lines were as follows: for Apigenin; 140 μ M, 50 μ M

and 50 μ M, for Kaempferol; 40 μ M, 100 μ M and 400 μ M, for Quercetin; 10 μ M, 60 μ M and 50 μ M, and for Luteolin; 50 μ M, 60 μ M and 75 μ M in Jurkat Clone E-6, MCF7 and HepG2 cell lines, respectively. According to the western blotting results in Jurkat cell line, Apigenin; in MCF7, Kaempferol; significantly ($p < 0.05$) reduced the NQO2 expression whereas in HepG2 cell lines any of them did not reveal any inhibitory effects.

Conclusion: As a result, the tested flavonoids were all inhibitors of NQO2 enzyme in nM levels and in Jurkat and MCF7 cell lines the cytotoxic effects of Apigenin and Kaempferol have been associated with NQO2 inhibition.

Keywords: NQO2, flavonoids, HepG2, MCF7, Jurkat

Özet: Amaç: Kinonlar bir veya iki elektron redüksiyonuna uğrayabilen çok reaktif maddelerdir. NQO1 ve NQO2 aracılığıyla bir elektron redüksiyonu aracılığıyla kararsız semiküinon radikalleri meydana gelir ve bunlar redoks siklusuna girerek, oksijen varlığında reaktif oksijen türevleri (ROS) oluşmasına sebep olurlar. NQO1 detoksifikasyondan sorumlu iken, NQO2 inhibitörlerinin antikanser etki gösterdiği bulunmuştur. Çalışmanın amacı doğal bazı bileşiklerin NQO2 inhibitörü olup olmadığının ve bu mekanizma

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üzerinden çeşitli kanser hücrelerinde etkili olup olmadıklarının araştırılmasıdır.

Metod: Öncelikle flavonoidlerin NQO2 enzim kinetiği üzerine etkileri araştırılmış ve Ki değerleri hesaplanmıştır. Daha sonra Jurkat Clone E-6, MCF 7 ve HepG2 kanser hücre hatlarında hücre canlılığı testi (MTT) üzerine etkileri ve western blotlama ile NQO2 proteinleri üzerine etkileri araştırılmıştır.

Bulgular: Çalışmamızda yer alan flavonoidlerin NQO2 inhibitör etkilerine ait Ki değerleri sırasıyla şu şekildedir: Apigenin (0.25 nM) < Kamferol (0.55 nM) < Kersetin (1.06 nM) < Luteolin (1.54 nM). Flavonoidlerin Jurkat Clone E-6, MCF7 ve HepG2 olmak üzere üç farklı hücre hattındaki sitotoksiteye ait IC₅₀ değerleri ise sırasıyla, Apigenin için 140 µM, 50 µM and 50 µM; Kamferol için 40 µM, 100 µM ve 400 µM; Kersetin için 10 µM, 60 µM ve 50 µM; Luteolin için 50 µM, 60 µM ve 75 µM şeklindedir. Jurkat hücre hattı için western blotlama sonuçları göre Kamferol anlamlı (p<0.05) bir şekilde NQO2 ekspresyonunu azaltırken; maddelerin hiçbirinde HepG2 hücre hattı için bu gözlenmemiştir.

Sonuç: Sonuç olarak test edilen tüm flavonoidlerin nM düzeyde NQO2 inhibitörü olduğu görülmüştür. Apigenin ve Kamferolün, Jurkat ile MCF7 hücre hatlarında göstermiş olduğu sitotoksik etki ise NQO2 inhibisyonu yapmalarıyla ilişkilendirilmiştir.

Anahtar Kelimeler: NQO2, flavonoidler, HepG2, MCF7, Jurkat

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Introduction

Quinones of polycyclic aromatic hydrocarbons are abundant in all burnt organic materials, including automobile exhaust, cigarette smoke and urban air pollution [1,2]. Quinones, including benzopyrene quinones and benzoquinones are highly reactive molecules and readily undergo either one- or two-electron reduction. One-electron reduction of quinones and their derivatives by enzymes, such as cytochrome P450 reductase (P450 reductase), ubiquinone oxidoreductase, etc. generate unstable semiquinones which undergo redox cycling in the presence of molecular oxygen. This leads to the formation of highly reactive oxygen species (ROS). The ROS and electrophiles

(semiquinones) that are generated by one-electron reduction of quinones cause oxidative damage, such as binding to DNA, lipid peroxidation and cytotoxicity. The various toxic effects, caused by exposure to quinones, eventually lead to hepatic, cardiovascular, nervous and renal tissue degeneration, apoptotic cell death, premature aging, cellular transformation and neoplasia [1,2].

The detoxification of quinones is catalyzed by a family of flavoproteins designated as the quinone oxidoreductases (NQOs or QRs). These molecules catalyze the two-electron reduction instead of one electron and detoxification of quinones and their derivatives [1].

NAD(P)H: quinone oxidoreductase 1 (NQO1) and NRH: quinone oxidoreductase 2 (NQO2) enzymes have unique ability to catalyze the two-electron reduction of quinones to hydroquinones, thereby prevents generation of toxic semiquinone radicals, reactive oxygen species and alkylation [3–6]. Recently expression levels or single nucleotide polymorphisms in NQO1 have been shown to be associated with numerous cancers, such as breast, prostate, colorectal, esophageal, gastric, osteocarcinoma, skin, acute myeloid leukemia (AML) as well as cardiovascular diseases, Parkinson's disease and diabetes. Correlatively, expression levels or single nucleotide polymorphisms in NQO2 are shown to be associated with AML, breast, prostate and esophageal cancer as well as cardiovascular diseases, Parkinson's disease and schizophrenia [4].

Recent studies have revealed that NQO2 differs from NQO1 in its cofactor requirement. NQO2 uses dihydronicotinamide riboside (NRH) rather than NAD(P)H as an electron donor. Another major difference between NQO2 and NQO1 is that NQO2 is resistant to typical inhibitors of NQO1 such as dicoumarol, cibacron blue and phenindone. Analysis of the crystal structure of NQO2 revealed that it contains a specific metal binding site, which is not present in NQO1. In addition to two-electron reduction of quinone compounds, NQO1 is involved in scavenging of superoxide anion radicals, maintenance of endogenous antioxidants, 20S proteasome pathway and stabilization of p53 protein. Owing to their broad substrate specificity, NQO1 and NQO2 play an important role in the detoxification of various endogenous and exogenous quinones, including estrogen quinones which are consistent with their association with many diseases [1,6].

Flavones, including quercetin and benzo(a)pyrene, are inhibitors of NQO2 [1,2]. However, structural requirements of flavones for the inhibition of NQO2 are different from those for NQO1. The most potent flavone inhibitor known for NQO2 is quercetin. Even though overlapping substrate specificities have been observed for NQO1 and NQO2, significant differences exist in relative affinities of

the various substrates. The role of NQO2 in detoxification of quinones remains unknown [2].

This project is designed to investigate the anticancer efficiencies of some NQO2 inhibitors like flavonoids such as Kaempferol, Apigenin, Luteolin and Quercetin via NQO2 (NRH or QR2) receptor in chronic myeloid leukaemia and some solid carcinoma tissues including hepatocarcinoma and breast cancer cells for the first time.

Material and Methods

Materials

Recombinant human NQO2 (rhNQO2) was purchased from Sigma, dissolved in 250 mM sucrose, and stored at -80°C . Dihydronicotinamide riboside (NRH) was prepared in Dr David Ross Lab (University of Colorado, Denver, USA) from NADH using previously reported methods [7,8]. Flavonoids were purchased from Sigma.

Kinetic studies

Mechanism-based inactivation of NQO2 by this flavonoid series was assayed using purified human recombinant NQO2 (rhNQO2). In these reactions (final volume of 0.5 mL),

rhNQO2 ($4\text{ }\mu\text{g/mL}$) was incubated with 0-50 nM flavonoid in the absence and presence of 25-250 μM NRH in 50 mM potassium phosphate buffer (pH 7.4) containing 5 μM FAD, 125 mM NaCl, and 1 mg/mL bovine serum albumin at room temperature. After 5 min, a 50 μL aliquot was removed and diluted 50-fold in stop buffer [50 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose, 5 μM FAD, 0.1% (v/v) Tween 20 and 0.25 mg/mL MTT]. An aliquot (960 μL) of the mixture was then transferred to a cuvette and mixed with 200 μM NRH and 10 μM menadione (final volume of 1 mL) and the linear increase in absorbance was monitored spectrophotometrically at 550 nm for 2 min at room temperature. NQO2 activity was measured as NRH-dependent menadione reductase activity using MTT as the final electron acceptor [9]. Reduction of the NRH by Apigenin, Kaempferol, Quercetin and Luteolin by reduced NQO2 was assayed at 450 nm. K_m and V_{max} values were calculated and K_i values were determined for each flavonoid by SigmaPlot (Version 12.0, Systat Software Inc., USA).

Cell culture

The cell lines used in the study were purchased from American Type Culture Collection (ATCC). Acute T cell leukemia cell line Jurkat, Clone E6-1 (ATCC Cat No. TIB-152); Human hepatocellular carcinoma cell line HepG2 (ATCC Cat No. HB-8065) and Human breast adenocarcinoma cell line MCF7 (ATCC Cat. No. HTB-22) were used in the present study.

Jurkat cells were maintained in RPMI 1640 Medium and HepG2 and MCF7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). Cell cultures were supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin and maintained under an atmosphere of 5% CO_2 with 90% relative humidity at 37°C according to standard protocols. The culture media were changed every other day.

Table 1: Michaelis-Menten and Lineweaver Burk calculated values for the kinetics of NQO2 catalysis.

Compounds	Concentration (nM)			
	0.0	0.5	5	50
Luteolin				
V_{max}	25.50	27.57	23.34	22.75
K_m	2.945	4.450	9.665	54.580
R^2	0.9955	0.9958	0.9966	0.9970
Kaempferol				
V_{max}	25.50	26.43	23.97	15.52
K_m	2.945	5.642	10.200	58.800
R^2	0.9955	0.9981	0.9859	0.9969
Quercetin				
V_{max}	25.50	29.20	27.60	11.50
K_m	2.945	15.720	21.590	75.790
R^2	0.9955	0.9546	0.9662	0.9568
Apigenin				
V_{max}	25.50	24.75	21.26	6.51
K_m	2.945	7.813	12.400	22.770
R^2	0.9955	0.9946	0.9913	0.9766

Western blotting analysis

The culture medium was discarded and the six-well plate was washed three times with PBS. The plate was added with the lysis buffer containing PMSF and incubated on ice for 30 min. Then, the lysates were collected and clarified by centrifugation at 12000 rpm for 10 min at 4°C and the supernatant was collected for protein analysis. The whole protein samples were boiled for 5 min in 5×loading buffer and separated using 10% SDS-PAGE. Proteins were transferred to PVDF membranes at a current of 300 mA for 90 min. The membranes were incubated in a blocking solu-

tion of 5% fat-free milk in Tris-buffered saline plus Tween 20 (TBST) for 1 h. The membrane was incubated overnight with a specific primary antibody. The membrane was then applied to a secondary antibody conjugated to HRP for 1 h. Following another triple washing, protein bands were visualized by ECL. Rabbit NQO2 (Santa cruz sc-32942) primary antibody (1:200) and rabbit β -tubulin (sc-9104) primary antibody (1:200) were used for the immunoblotting procedure. Goat antirabbit secondary antibody (Jackson Immunoresearch 111-035-144) (1:10.000).

Statistical analysis

The data were expressed as mean $\pm$ SEM and a statistical analysis was performed by one-way ANOVA post-hoc Tukey. The results were considered statistically significant at $p < 0.05$.

Results

Kinetic studies

The K_m unit for reduction of Luteolin, Kaempferol, Quercetin and Apigenin were determined to be 2.5, 1.9, 6.0 and 1.9 nM respectively for NQO2 mediated catalysis of CB1954 (5-aziridin-1yl)-2,4-dinitrobenzamid) (Table 1, Figure 1 and Figure 2). Enzyme catalyzed reaction, the graph of velocity against substrate concentration was non-linear for all above assays. When compared among themselves the rates of the catalysis were in following order Quercetin>Luteolin>Kaempferol=Apigenin. The tested flavonoids were all competitive inhibitors of NQO2 enzyme in nM levels.

K_i values for NQO2 inhibitory effects of the studied flavonoids are respectively, Apigenin (0.25 nM)<Kaempferol (0.55 nM)<Quercetin (1.06 nM)<Luteolin (1.54 nM).

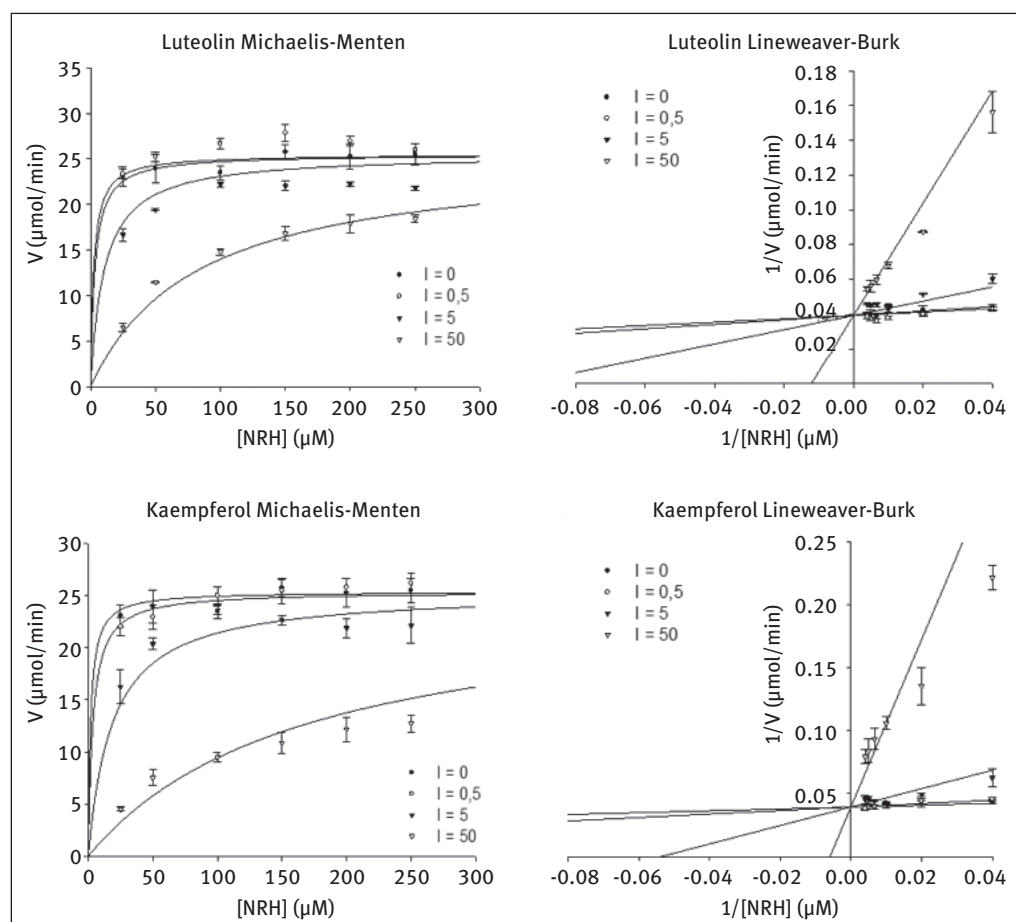


Figure 1: Michaelis-Menten and Lineweaver Burk plots for the kinetics of NQO2 catalysis. Reduction of Luteolin and Kaempferol by NQO2 enzyme, respectively.

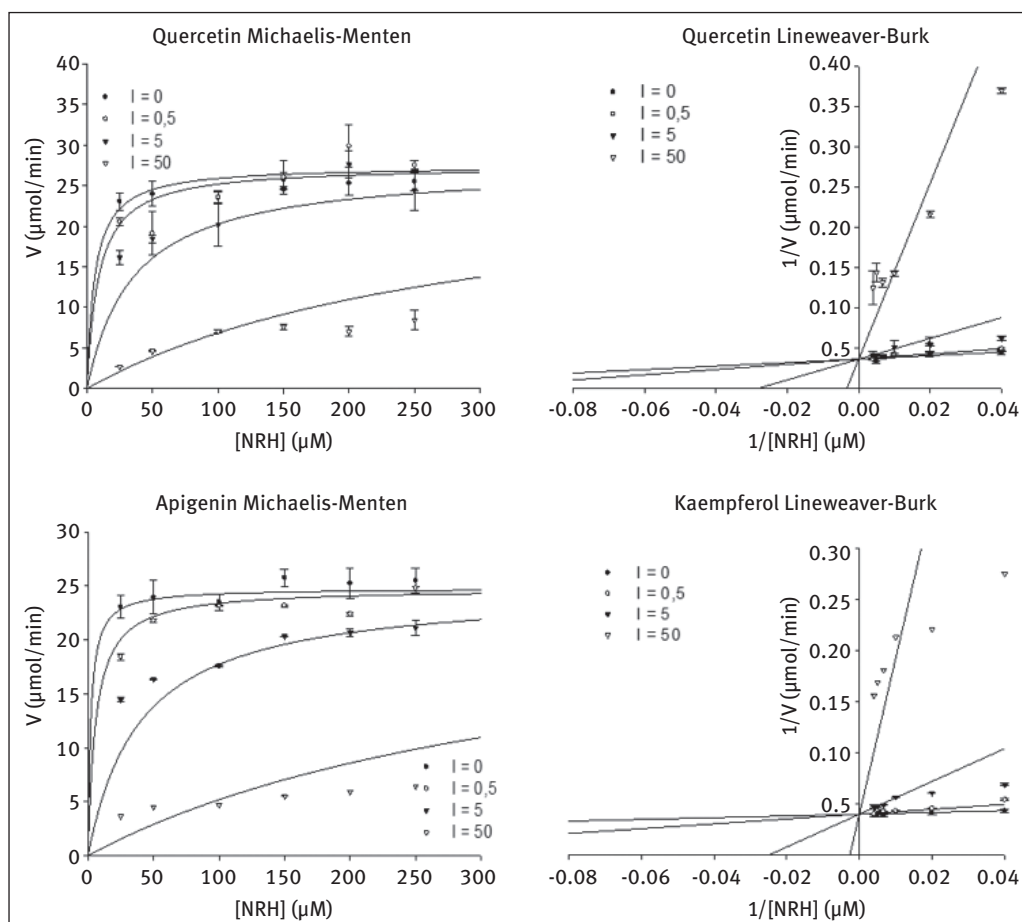


Figure 2: Michaelis-Menten and Lineweaver Burk plots for the kinetics of NQO2 catalysis. Reduction of Quercetin and Apigenin by NQO2 enzyme, respectively.

Cell viability results

The cell lines used for the cell viability assays are Jurkat Clone E-6, MCF7, HepG2. As a result of MTT assay, the IC_{50} values for flavonoids in three different cell lines were shown in Table 2.

In Jurkat Clone E-6 cell line, Quercetin (10 μM); in MCF7 cell line, Apigenin (50 μM); in HepG2 cell line, Apigenin (50 μM) and Quercetin (50 μM) had the lowest IC_{50} levels.

Western blotting analysis

According to the western blotting results in Jurkat cell line, Apigenin (Figure 3 and 4); in MCF7, Kaempferol (Figure 5 and 6); significantly ($p < 0.05$) reduced the NQO2 expression whereas in HepG2 cell lines any of them did not reveal any inhibitory effects (Figure 7, 8 and 9). Melatonin was loaded as a positive control. Proteins were analysed by using Image J software (Version 1.48, NIH, USA).

Discussion

It has been well documented that alkyl phenol group present in endogenous molecules, environmental toxicants, carcinogens and chemotherapeutic drugs could undergo enzymatic oxidation by peroxidases and P450 enzymes to form transient radical species which can further oxidize to quinones [10]. Quinones are known electrophilic species that covalently react with cellular macromolecules to form DNA adducts and/or protein conjugates [10–14]. The covalent binding of quinones has been shown to lead to various forms of cytotoxicity, immunotoxicity and carcinogenesis [10–14].

On the contrary, the distribution of NQO1 and NQO2 in many tumors could have a potential impact on the development and efficacy of quinone generating or containing anticancer agents. Many solid tumors including thyroid, adrenal, breast, ovarian, cornea, colon, liver and non-small cell lungs cancers express the NQO1 gene. Expression of the NQO2 gene is high in normal human liver and

skeletal muscle [1,2,15] and minimal in the kidney, heart, pancreas, brain and red blood cells [16]. There is growing interest in the possible use of flavonoids containing molecules as promising chemotherapeutic drugs [10]. Recently, numerous flavonoids have been developed as probable antitumor agents [10,17]. The ability of NQO2 to reduce flavonoids may have significant implications against the efficacy of potential flavonoid based anticancer drugs currently under development.

These findings may also have broad implications for the efficacy of potential flavonoids based anticancer drugs that are currently under development.

Several structurally diverse compounds have been shown to be competitive inhibitors of NQO2, including the natural products resveratrol [18,19], quercetin [9], casimiroin [20] and melatonin [21,22] and the drugs chloroquine [16,23] and imatinib [24,25]. In this study we have demonstrated the NQO2-catalyzed reduction of the flavonoids Apigenin, Luteolin, Kaempferol and compared with the known inhibitor Quercetin and determined the Ki values for these

Table 2: The IC₅₀ values for tested flavonoids in Jurkat Clone E-6, MCF7 and HepG2 cell lines.

Cell line/ Flavonoids	Apigenin	Kaempferol	Quercetin	Luteolin
	μM			
Jurkat Clone E-6	140	40	10	50
MCF7	50	100	60	60
HepG2	50	400	50	75

flavonoids for the first time. According to our results all the flavonoids were competitive inhibitors of NQO2 in nM levels. The presence of additional hydroxyl groups on C3 and B3' positions significantly decreases the ability to inhibit NQO2. The differences in the inhibition profiles of flavones are related with hydroxyl groups (Figure 10). Detailed explanation of the interaction between NQO2 and flavonoids requires examination the X-ray crystal and NMR analyses of the complex of NQO2 and flavonoids.

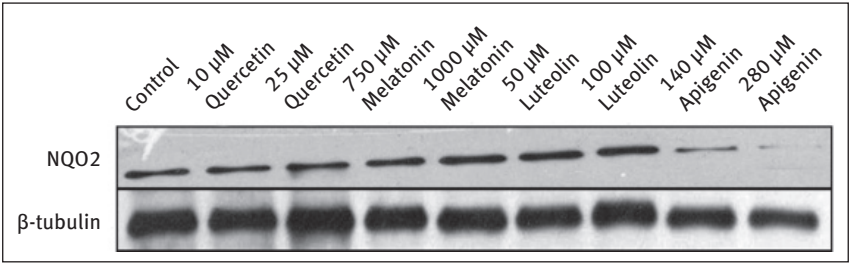


Figure 3: NQO2 expression after treatment with IC₅₀ doses of tested flavonoids in Jurkat cell line.

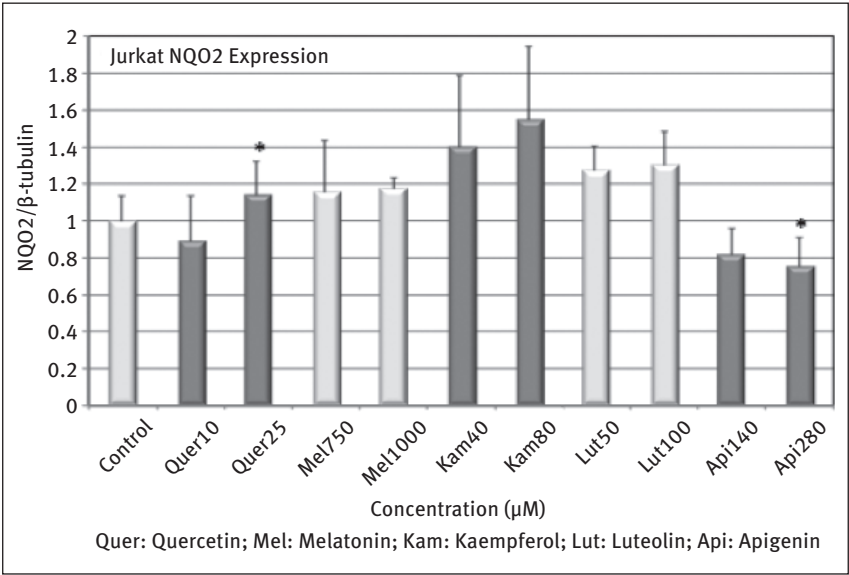


Figure 4: NQO2 expression after treatment with IC50 doses of tested flavonoids in Jurkat cell line (Image J analysis).

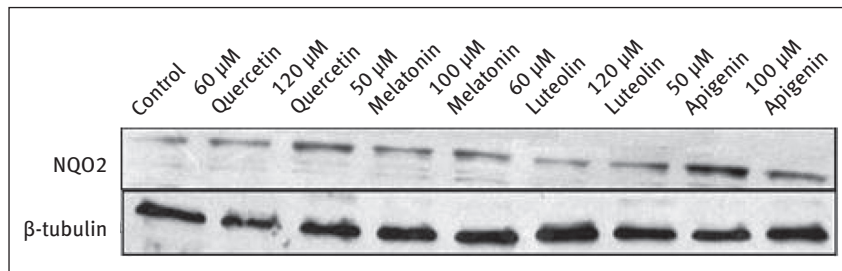


Figure 5: NQO2 expression after treatment with IC_{50} doses of tested flavonoids in MCF7 cell line.

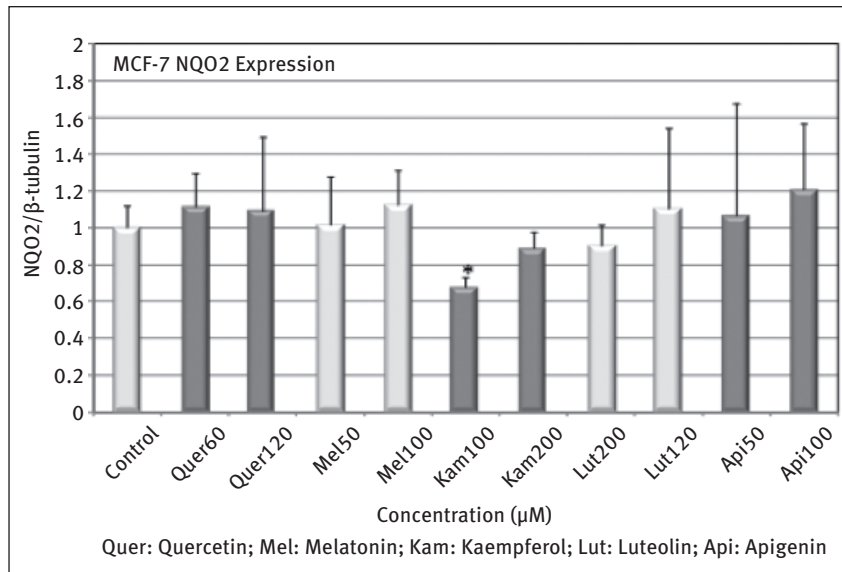


Figure 6: NQO2 expression after treatment with IC_{50} doses of tested flavonoids in MCF7 cell line (Image J Analysis).

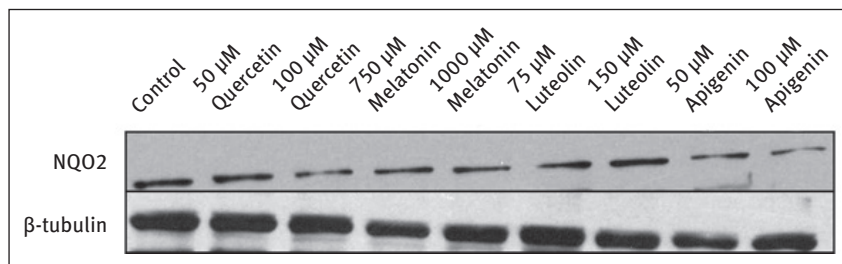


Figure 7: NQO2 expression after treatment with IC_{50} doses of tested flavonoids in HepG2 cell line.

Even though to date the biological role of NQO2 is poorly understood, there are studies showing evidence for its involvement in skin carcinogenesis. The most prominent example is that NQO2 knockout mice are far more prone to develop skin tumors after the topical application of carcinogens [26]. More recently, inhibition of melanoma cell growth and clonogenicity was correlated with upregulation of NQO2 and p53 [27]. In this context, the parallel inhibition of cell growth induced by melatonin, the

natural ligand of NQO2, suggests that expression of NQO2 might be another factor determining the oncostatic effects of melatonin [28]. In our study, when we compare the IC_{50} levels of the flavonoids in different cell lines revealed that in Jurkat Clone E-6 cell line, Quercetin (10 μ M); in MCF7 cell line, Apigenin (50 μ M); in HepG2 cell line, Apigenin (50 μ M) and Quercetin (50 μ M) had the lowest IC_{50} levels but HepG2, a primary hepatoma cell line, it is more resistant to flavonoids than other cell lines. Toxic substances

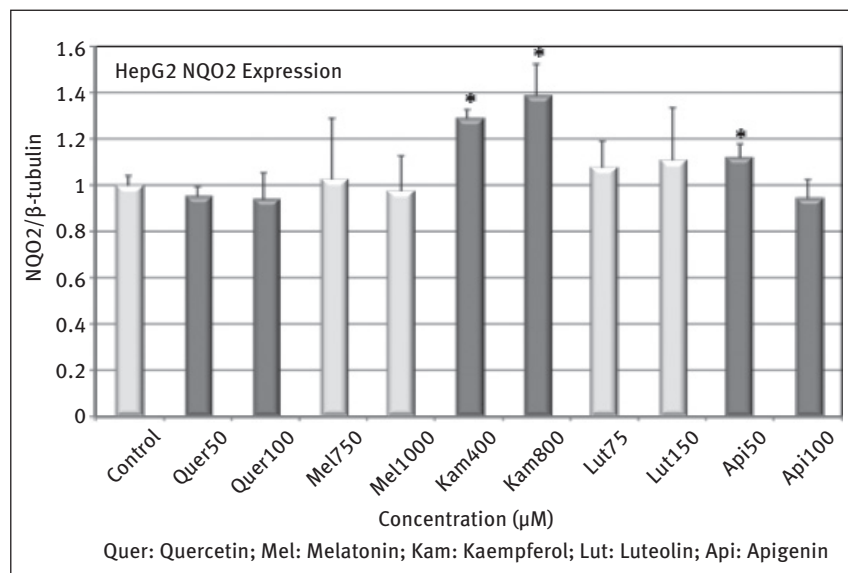


Figure 8: NQO2 expression after treatment with IC_{50} doses of tested flavonoids in HepG2 cell line (Image J Analysis).

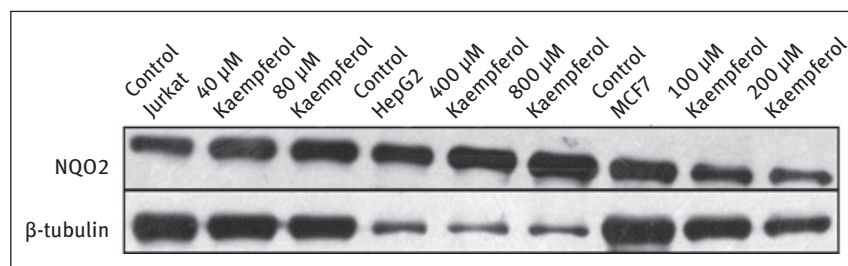


Figure 9: NQO2 expression after treatment with IC_{50} doses of Kaempferol in Jurkat, HepG2 and MCF7 cell lines.

are eliminated by liver which is the major organ of xenobiotic metabolism. Liver cells contain several detoxification enzymes, such as phase I and II, for proving toxicant elimination and detoxification [29]. Furthermore, when we compare the toxicity effects of flavonoids on cell lines, it reveals that Kaempferol is less toxic than other flavonoids on HepG2 and MCF7 cell lines. These results show that the competitive inhibitors of NQO2 enzyme have cytotoxic effects on different cell lines on μM levels.

Furthermore, according to our western blotting results, in Jurkat cell line, Apigenin (Figure 3); in MCF7, Kaempferol (Figure 5); significantly ($p < 0.05$) reduced the NQO2 expression whereas in HepG2 cell lines any of them did not reveal any inhibitory effects (Figure 7 and 9) and apigenin is the most potent molecule in terms of Jurkat NQO2 protein expression and *in vitro* NQO2 kinetic inhibition. These results reveal that in different cell lines different flavonoids might show their cytotoxic effect via NQO2 inhibition.

In conclusion, the tested flavonoids were all inhibitors of NQO2 enzyme in nM levels and in Jurkat and MCF7

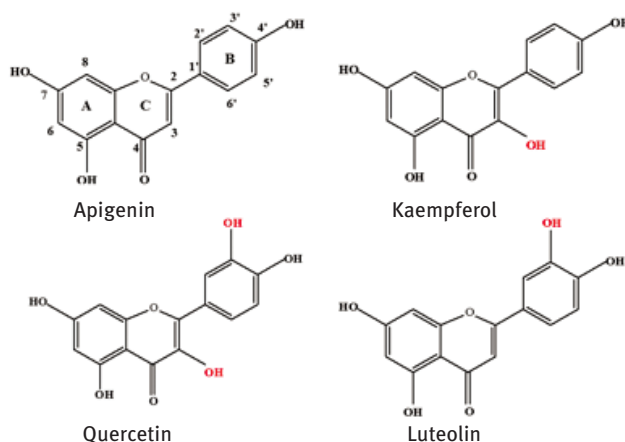


Figure 10: Chemical structures of apigenin, quercetin, kaempferol and luteolin.

cell lines the cytotoxic effects of Apigenin and Kaempferol have been associated with NQO2 inhibition.

Since there is also heterogeneity across different melanoma cell lines, more work is needed to fully characterize the factors determining dependency of NQO2 inhibition and tumor behavior and future studies are required to understand the inhibition and regulation of NQO2 expression, its structure–function relationship with the tested flavonoids and NQO2's role in cancer treatment.

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