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Antioxidant Capacity and Radical Scavenging Activity of *Silybum marianum* and *Ceratonia siliqua*

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Abstract

The aim of this study is to investigate the protective effects of *Silybum marianum* and *Ceratonia siliqua* extracts on unsaturated fatty acids and the suppressive effects on the Fenton reagent in lipid peroxidation (LPO). The LPO levels in the *Silybum marianum* and *Ceratonia siliqua* groups were higher than the control group ($p<0.001$), whereas, these levels were lower by about 50 % than that of the Fenton R group ($p<0.001$). In addition, it was determined that *S. marianum* and *C. siliqua* extracts had scavenging effect on the DPPH radical as it is in quercetin ($p>0.05$). The fatty acid levels in the Fenton R group were lower than that of the control group ($p<0.001$). However, fatty acid levels in the *S. marianum* and *C. siliqua* groups were higher than the Fenton R group ($p<0.001$). The Linoleic acid level in the *S. marianum* group was slightly lower than the control group ($p<0.01$) but the oleic acid level was higher than the control group ($p<0.001$). Our results confirm that extracts of *S. marianum* and *C. siliqua* decreased the LPO level and protected markedly the unsaturated fatty acids against free radicals.

Keywords: *Ceratonia siliqua*, Fenton reagent, lipid peroxidation, radical scavenging effects, *Silybum marianum*.

Silybum marianum and _Ceratonia siliqua_'nın Antioksidan Kapasitesi ve Radikal Koruyucu Aktivitesi

Özet

Bu çalışmanın amacı *Silybum marianum* ve *Ceratonia siliqua* ekstraktlarının doymamış yağ asitleri üzerinde koruyucu ve lipid peroksidasyonunda Fenton reaktifini bastırıcı etkisinin incelenmesidir. *Silybum marianum* ve *Ceratonia siliqua* gruplarında lipid peroksidasyon seviyesi (LPO) kontrol grubundan yüksekken ($p<0,001$), *S. marianum* ve *C. siliqua* gruplarında LPO seviyesinde Fenton reaktifi grubuna göre %50 oranında azalma gözlenmiştir ($p<0,001$). Buna ilaveten, *S. marianum* ve *C. siliqua* ekstraktlarının DPPH radikali üzerinde kersetin kadar ($p>0,05$) süpürücü etkiye sahip olduğu belirlenmiştir. Yağ asidi seviyeleri Fenton reaktifi grubunda kontrol grubundan daha düşüktür ($p<0,001$). Bu nedenle, *S. marianum* ve *C. siliqua* gruplarında yağ asidi seviyeleri Fenton reaktifi grubundan daha yüksektir ($p<0,001$). *S. marianum* grubunda, linoleik asit seviyesi, kontrol grubundan kısmen daha düşüktür ($p<0,01$) fakat oleik asit seviyesi, kontrol grubundan daha yüksektir ($p<0,001$). Sonuçlarımız bitki ekstraktlarının LPO seviyesini azaltıp, serbest radikallere karşı doymamış yağ asitlerini belirgin şekilde koruduğunu desteklemiştir.

Anahtar Kelimeler: *Ceratonia siliqua*, Fenton reaktifi, lipid peroksidasyonu, radikal süpürücü etki, *Silybum marianum*

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INTRODUCTION

Oxidative stress may be a key factor in the onset of certain diseases, including cancer (Devasagayam 1995). Oxy-radicals play important roles in the initiation, promotion, and progression of carcinogenesis (Devasagayam 1995, Duthie 1997, Kennedy 1997). It is considered that a significant event in oxy-radical-mediated carcinogenesis is the extensive oxidative damage to the nuclear membrane (Stinson 1992), which leads to deoxyribonucleic (DNA) damage such as DNA single-strand breaks and possibly facilitation of carcinogenesis (Devasagayam 1995, Duthie 1997, Kennedy 1997). To prevent cellular damage leading to cancer caused by oxy-radicals, the level of tissue antioxidants is critical (Duthie 1997, Di Carlo 1999).

Interest in natural sources of antioxidant molecules for use in the food, beverage and cosmetic industries has resulted in a large body of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Of these substances, the phenolic compounds, which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (Hirano 2001).

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Silymarin is isolated from the fruits and seeds of the milk thistle (*Silybum marianum*) and in reality is a mixture of three structural components: silibinin, silydianine, and silychristine. Milk thistle is a member of the *Asteraceae* family (Kren and Walterovab 2005). It has been reported as having multiple pharmacological activities including antioxidant, hepatoprotectant and anti-inflammatory agent, antibacterial, antiallergic, antimutagenic, antiviral, antineoplastic, antithrombotic agents, and vasodilatory actions (Abascal and Yarnell 2003). Asghar et al. (2008) suggested that *silymarin* may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement.

The carob tree (*Ceratonia siliqua* L.) is widely cultivated in the Mediterranean countries (Battle and Tous 1997). The fruit of the carob tree is a brown pod 10-25 cm in length. The two principal components of the carob fruit are the pulp and seed. The important ingredient of the seeds is galactomannan which is known for its thickening effects and is widely used in the food industry (Marakis 1996). The main application of carob pods is animal feed production, but in a few countries the pods are also used as a cocoa substitute (Kumazawa 2002). Carob pods contain lots of polyphenols, especially highly condensed tannins. A phenolic analysis revealed high contents of different forms of gallic acid (free gallic acid, gallotannins, and methyl gallate) and large amounts of quercetin and myricetin derivatives (Owen et al. 2003, Papagiannopoulos et al. 2004). Thus, carob fiber combines two positive nutritional ingredients, namely polyphenols and dietary fiber. Recent studies discovered that carob fiber has cholesterol lowering activities in persons suffering from hypercholesterolemia (Zunft et al. 2001, 2003). There are other reported antioxidants properties in different *in vitro* test systems (Haber 2002).

The antioxidant influence of plants for health and quality of life is important. The aim of this study was to prevent LPO resulting from the OH• radical in the *in vitro* environment related to the flavonoid ingredient of the *S. marianum* and *C. siliqua* L. used by people, to detect the antioxidant capacity of the DPPH radical by determining its scavenging features, and to detect its preventive influence on unsaturated fatty acids according to the LPO preventive characteristic.

MATERIAL AND METHOD

Chemicals

The following; oleic (18:1, n-9), linoleic (18:2, n-6), linolenic acid (18:3, n-3), Tween 20, tris-hydrochloride quercetin, myricetin, resveratrol, cathechin, naringin, naringenin, kaempferol, HPLC grade methanol, acetonitrile, n-hexane, isopropyl alcohol, $FeCl_2$, H_2O_2, KH_2PO_4, butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), n-butanol, α,α-diphenyl-β-picryl-hydrazyl (DPPH•), dimethyl sulfoxide (DMSO), and ethyl alcohol were purchased from Sigma-Aldrich.

Preparation of the Plant Extracts

The *Silybum marianum* and *Ceratonia. siliqua* L. samples are sold by the herb and spice seller in Elazıg as dehydrated and powdered in the bray. 1 g herb material was homogenized in a 10 mL solution of 80% methanol. Homogenates were them centrifuged at 5000 rpm at +4°C. After centrifugation, the flavonoid analysis of the supernatant was carried out using HPLC equipment. Then, the methanolic extract was concentrated and dried in a vacuum at 50°C using a rotary evaporator. Each extract was then re-suspended in DMSO as a stock solution.

Antioxidative Activity Testing of Plant Extracts

Antioxidative activities of the *S. marianum* and *C. siliqua* extracts were determined by the method of Shimoi et al. (1994) with the following modifications. The Fe^{++} ($FeCl_2$ $2H_2O$) and hydrogen peroxide solutions were prepared fresh for every treatment using twice deionized water. Extracts of the *S. marianum* and *C. siliqua* were also prepared fresh using DMSO. Oleic acid (3.35 mM), linoleic acid (9.01 mM), and linolenic acid (2.30 mM) were dissolved in the DMSO.

During the *in vitro* experiment, the first group was used as a control (n=5), the second group was the Fenton reagent group (n=5), the third group was *S. marianum* extract group (n=5), and the fourth group was the *C. siliqua* group (n=5).

The control group was prepared with 0.5 mL of fatty acid and a buffer solution (0.2% Tween 20 / 0.05 M Tris•HCl / 0.15 M KCl, pH 7.4). The Fenton R group was prepared with 0.5 mL of fatty acid, a buffer solution, $FeCl_2$ (50 μM), and hydrogen peroxide (0.01 mM). The *S. marianum* group was prepared with 0.5 mL of fatty acid, a buffer solution, $FeCl_2$ (50 μM), hydrogen peroxide

(0.01 mM), and 1 mL *S. marianum* extract. The *C. siliqua* group was prepared with 0.5 mL of fatty acid, a buffer solution, $FeCl_2$ (50 μM), hydrogen peroxide (0.01 mM), and 1 mL *C. siliqua* extract.

After incubation of the mixture at 37°C for 24 h, 100 μL of a 4% (w/v) BHT solution was added to prevent further oxidation. Then, 1 mL was taken each mixture and 1 mL of 0.6% TBA was added to the reaction mixture and incubated at 90°C for 45 min. Samples were allowed to cool to room temperature and the pink pigment produced was extracted with 3 mL of n-butanol. Samples were then centrifuged at 6000 rpm for 5 min and, the concentration of the upper butanol layer was measured by HPLC- fluorescence detector.

Quantification of LPO Level *in vitro* Environment

The products of the peroxidation of fatty acids in the reaction environment was determined by reading the fluorescence detector set at λ (excitation)= 515 nm and λ (emission)= 543 nm. Formation of the malonaldehyde in vitro environment was expressed as thiobarbituric acid-reactive substances (TBARS) calculated from a calibration curve using 1, 1, 3, 3-tetraethoxypropane as the standard. The MDA-TBA complex was analyzed using HPLC equipment. The equipment consisted of a pump (LC-10 AD_{VP}), a Fluorescence detector (RF–10 $_{XL}$), a column oven (CTO-$10AS_{VP}$), an autosampler (SIL-$10AD_{VP}$), a degasser unit (DGU-14A), and a computer system with class *VP* software (Shimadzu, Kyoto Japan). An Inertsil ODS-3 column (15×4.6 mm, 5 μm) was used as the HPLC column. The column was eluted isocratically at 20ºC with a 5 mM sodium phosphate buffer (pH=7.0) and acetonitrile (85:15, v/v) at a rate of 1 mL/min (de las Heras et al. 2003).

Quantification of Remaining Fatty Acids in the *in vitro* Environment

Remaining mixtures of oleic (18:1 n-9), linoleic (18:2 n-6) and linolenic (18:3 n-3) acids in the test tube were converted to methyl esters by using 2 % sulfuric acid (v/v) in methanol (Christie 1992). The fatty acid methyl ester forms were extracted with n-hexane. Analysis was performed in a Shimadzu GC-17A instrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m 0.25 mm i.d. Permabond fused-silica capillary column (Machery- Nagel, Germany). The oven temperature was programmed for 160-215°C at 4°C / min. Injector and FID temperatures were 240 and 280°C, respectively. The nitrogen carrier gas flow was 1 mL/min. The methyl esters of oleate, linoleate, and linolenate were identified by comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10 software version 2.01 was used to process the data. The results were expressed as μmol/mL.

Antioxidant Assay by DPPH Radical Scavenging Activity:

The free radical scavenging effect in the extracts was assessed by the decoloration of a methanolic solution of DPPH• according to the method of Liyana-Pathiranan and Shahidi (2005). A solution of 25 mg/L DPPH in methanol was prepared and 4.0 mL of this solution was mixed with 50 μL of extract in DMSO. The reaction mixture was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Quercetin was used as references. The absorbance at 517 nm was measured after reaction in the dark at 30°C for 30 min. Lower absorbance of the reaction mixture indicates high free radical scavenging activity. All tests were performed in triplicate, and the percentage inhibition of the radicals due to the antioxidant properties of the extracts were calculated as shown below

DPPH radical scavenging activity (%)= [(Abs control – Abs sample)]/ (Abs control)] x 100

Where Abs control is the absorbance of the DPPH radical + methanol; the Abs sample is the absorbance of the DPPH radical + sample extract /standard.

Chromatographic Conditions for Flavonoid Analysis:

Chromatographic analysis was carried out using a PREVAIL C18 reversed-phase column (15x4.6mm, 5μm), the mobile phase was methanol/water/acetonitrile (46/46/8, v/v/v) containing 1.0% acetic acid (Zu et al. 2006). This mobile phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated ultrasonically prior to use. Catechin (CA), Naringin (NA), Rutin (RU), Resveratrol (RES), Myricetin (MYR), Morin (MOR), Naringenin (NAR), Quercetin (QU), and Kaempferol (KA) were quantified by DAD following RPHPLC separation at 280 nm for CA and NA, 254 nm for RU, MYR, MOR and QU, 306 nm for RES, and 265 nm for KA. Flow rate and injection volume were 1.0 mL/min and l0 μL,

respectively. The chromatog-raphic peaks of the analyses were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using an external standard method. All chromatographic operations were carried out at 25°C.

Statistical analysis:

The experimental results were reported as mean ± SEM. Statistical analysis was performed using SPSS Software. Analysis of variance (ANOVA) and an LSD test were used to compare to the plant extract groups with the Fenton R group.

RESULTS

The present study showed that MDA-TBA levels were significantly high in the groups with the plant extract compared with the control group ($p<0.001$) whereas, these levels were about 50% lower than that of the Fenton R group ($p<0.001$) (Figure1). The difference between plant extracts and quercetin was statistically non-significant ($p>0.05$) when they were evaluated in terms of DPPH capacity of free radical scavenging. Additionally, both plant extracts were detected to have an effect of high radical scavenging as quercetin (Table1).

According to the results of the gas chromatog-raphic analysis of fatty acids, the amount of oleic, linoleic, and linolenic acid were significantly high in the groups with the plant extract in comparison to the Fenton R. group ($p<0.001$) (Fig. 2). A significant decrease in the Fenton R. group compared to the control group was observed ($p<0.001$) (Fig. 2). It was shown that each fatty acid in the groups with plant extract significantly repressed oxidation compared to the Fenton R group ($p<0.001$) (Fig. 2). The Linoleic acid levels in the S. marianum group were slightly lower than the control group ($p<0.01$) but the oleic acid levels were higher than the control group ($p<0.001$) (Fig. 2). According to the flavonoid analysis results by HPLC-DAD, a significant amount of catechin, myrcetin, quercetin, naringin, naringenin, resveratrol and catechin, naringenin, naringin, and quercetin were present in the extracts of *S. marianum* and *C. siliqua*, respectively (Table 2).

DISCUSSION

The importance of ROS and free radicals has attracted increasing attention over the past decade. ROS, which include free radicals such as superoxide anion radicals (O2•−), hydroxyl radicals (OH•) and non-free-radical species such as H_2O_2 and singled oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and the aging process (Halliwell and Gutteridge 1989, Gülçin et al. 2002 a, b).

Table 1. The DPPH• Scavenging effects of S.marianum and *C.siliqua* methanolic extracts (50 μL)

Groups	Absorbance of 0 the minute	Absorbance of 30 the minutes	DPPH Scavencing effect Results (%)
DPPH Solution (S)	0,6	0,6	-
DPPH S+*S.marianum*	0,56	0,020	94,94±0,62[a]
DPPH S+ *C.siliqua*	0,55	0,030	93,81±0,44[a]
DPPH S+ *Quercetin*	0,55	0,003	99,52±0,52[a]

[a]$p>0.05$

Table 2. The contents of flavonoids in the extracts of *S.marianum* and *C.siliqua* (μg/1 g)

	S.marianum	*C.siliqua*
Quercetin	39,75	2
Catechin	373	6338
Naringin	12	10,25
Naringenin	10,5	45
Total	435,25	6395,25

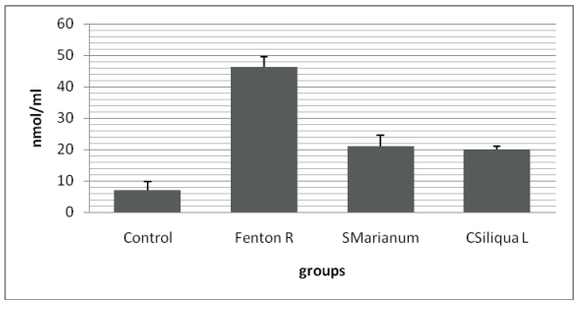

Fig. 1. The levels of MDA-TBA in vitro environment of the groups

Note: Bar diagrammatic representations of MDA-TBA concentrations in different groups . Each bar represents means ±SEM (n=5).

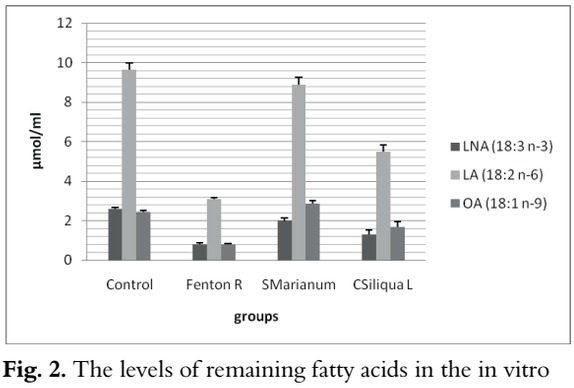

Fig. 2. The levels of remaining fatty acids in the in vitro environment (μmol/ml)

Note: Bar diagrammatic representations of fatty acids, oleic, linoleic and linolenic acid amounts with gas chromatograph. Each bar represents means ±SEM (n=5).

Our findings showed that the levels of MDA-TBA, which is one of the most important criteria of LPO (Kose et al. 2010, Sahan et al. 2010), is significantly high in the plant extract groups compared with the control group ($p<0.001$) in the *in vitro* environments. However, this increase was found to be 50% lower than that of the Fenton R group ($p<0.001$) (Figure1).

In this study, the reduction of the elevated LPO levels which was determined only in the Fenton containing group is believed to be related to its flavonoid ingredient. The flavonoid ingredients of *S. marianum* consist of catechin, myrcetin, quercetin, naringin, naringenin, resveratrol, rutyn, and those of *C. siliqua* were catechin, naringenin, naringin, and quercetin (Table 2).

Flavonoids have a number of biological effects *in vivo* and *in vitro* (Shimoi 1994, Di Carlo 1999). Several studies have reported the antioxidant activity of plant extract and their relationship with the phenolic compound content (Aaby 2004, Silva 2005, Sun 2005, Yuan 2005, Singh 2007).

Yousuf et al. (2009) assessed that the mitochondrial LPO level decreased with resveratrol treatment on rats. Kostiuk et al. (1988) reported that the antioxidative activity of quercetin and rutin in various systems of lipid peroxidation. Quercetin, with an *o*-di-hydroxyl structure in ring B, could directly scavenge free radicals effectively as reported by Gao et al. (2000). Also, naringenin and catechin have the same molecular structure as quercetin. It has been reported that flavonoids may act as scavengers of the hydroxyl radical by acting as chain breaking antioxidants, and as hydrogen donors with the formation of a less reactive flavonoid (aroxyl-ArO) radical ($ArOH + \bullet OH \rightarrow ArO\bullet + HOH$) (Qu et al. 2002).

Zou et al. (2000) demonstrated that resveratrol protected LDL against both Cu (2+)-induced and azo compound-initiated oxidative modification in vitro, which might be due to its free radical scavenging capacity. In addition, Belguendouz et al. (1997) assessed that transresveratrol was less potent than flavonoids (but more than trolox) as a scavenger of free radicals.

Hancock et al. (2006) demonstrated that resveratrol attenuated EtOH-induced damage. Sengottuvelan et al. (2009) indicated that 1, 2-dimethylhydrazine-induced DNA damage and oxidative stress was suppressed/ prevented effectively by chronic resveratrol supplementation.

We propose that there is a link between the decrease in the LPO level of the plant extracts and scavenging capacity of the DPPH radical. When the DPPH free radical scavenging capacity was examined, it was observed that there is a statistically insignificant difference between *Silybum marianum* vs quercetin and *Ceratonia siliqua* vs quercetin and they both have a high level of DPPH radical scavenging ability similar to quercetin (Table1). The test for antioxidant activity against the DPPH-radical demonstrated marked antioxidative properties of substances produced by the culture of *Silybum marianum* (Tümova et al. 2004). Psotová et al. (2002) have suggested that in contrast to quercetin, silybin, silydianin, and silychristin did not chelate iron in an aqueous solution. The results suggest that silymarin may prevent doxorubicin-mediated damage to the rat heart membrane primarily through a free radical scavenging mechanism.

In this study, it was determined that the levels of the three fatty acids in the extracts containing *Silybum marianum* and *Ceratonia siliqua* were high compared to the Fenton R group (Fig. 2). For this reason, we suggest that the flavonoids of these extracts may protect unsaturated fatty acids from attacks of free radicals.

Pekkarinen et al. (1999) reported that the antioxidant activity of the flavonoids quercetin, myricetin, kaemferol, catechin, and rutin on methyl linoleate oxidation. They reported that the antioxidant activity of flavonoids in the methyl linoleate system is due to radical scavenging activity and flavonoids may effect the decomposition of hydroperoxides.

Määttä-Riihinen et al. (2005) showed that silymarin and silibinin, likely through antioxidant and free radical scavenging mechanisms, inhibit the generation of oxLDL and oxidation-specific neoepitopes recognized by scavenger receptor and Fc gamma receptors expressed on monocytes /macrophages.

Based on experimental results, we observed that both plant extracts decreased the LPO levels, and they also had high capacity level to scavenge the radical of DPPH.

As a result, we suggest that there is a strong link between the flavonoid ingredients of *Silybum marianum* and *Ceratonia siliqua* and their ability in

suppression of lipid peroxidation.

If one considers the fact that unsaturated fatty acids are also protected from radical sourced oxidation, it is possible to argue that those plants have high level of antioxidant activities. Therefore, these plants could be evaluated as rich sources of natural antioxidants and used in the pharmacological and food industry because of their antioxidant properties.

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