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Antioxidant Activity of Flaxseed (*Linum usitatissimum* L.) shell and Analysis of Its Polyphenol Contents by LC-MS/MS

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Abstract: Flaxseed (*Linum usitatissimum* L.) is important source of oil and protein for industrial, pharmaceutical, and nutritional applications. In order to estimate the effects of lyophilized aqueous extract of flaxseed shell (AEF) and evaporated ethanolic extract of flaxseed shell (EEF), we studied their DPPH, ABTS, DMPD and $O_2^{\bullet-}$ scavenging effects. Total antioxidant activity by ferric thiocyanate method, Fe^{3+}, Cu^{2+} and $[Fe^{3+}$-$(TPTZ)_2]^{3+}$ reducing ability, and Fe^{2+} chelating activity. Also, α-tocopherol, BHA, trolox, and BHT were used as positive controls. The results clearly AEF and EEF demonstrated effective antioxidant activity. The quantity of *p*-hydroxybenzoic, vanillin, *p*-coumaric acid, ascorbic acid, ferulic acid, and ellagic acid were investigated by LC-MS/MS. The present study will introduce a novelty for further studies on the antioxidant effects of AEF and EEF.

Keywords: Flaxseed; *Linum usitatissimum*; antioxidant activity; polyphenol content

1. Plant Source

Flaxseed (*Linum usitatissimum* L.) is an annual herb belonging to the Linaceae family obtained from local market of Erzurum in 2011. This plant has been cultivated for oil, fiber, and food because of its beneficial chemical composition. The plant material was identified by Prof. Dr. Yusuf Kaya, Ataturk University, Erzurum, Turkey.

2. Previous Studies

Plant materials contain a lot of bioactive phenolics, which demonstrate biological activity including antiradical, antioxidant, antimicrobial and anticancer effects. These chemical classes have many beneficial health effects and prevent some chronicle diseases [1]. Antioxidants are synthetic or natural compounds that can delay some types of cell damage and quench reactive radical species formed during oxidative reactions in metabolism. They include mainly phenols, polyphenols, carotenoids, anthocyanins and tocopherols, which main groups of phytochemicals found in plants [2]. The basic structure of phenol contains a hydroxyl group (-OH) linked to the aromatic ring. The biological activates of phenolic compounds differ depending on the position and number of phenolic groups and their locations [3]. Vegetables and fruits are main resources of naturally occurred antioxidants and reduce risks of certain diseases. Natural antioxidants have been associated with cancer development are generally opted by consumers because of their safety [4]. Therefore, the identification and the corresponding importance of natural antioxidant sources need to be researched from the standpoint of health-improving properties [5]. On the other hand, it was reported that support

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the usage of natural antioxidant additives decrease oxidative stress level and chronic disease [6]. Also, it was reported that synthetic antioxidants have shown side effects including mutagenic, carcinogenic and toxic impacts. For this reason, the usage of these synthetic compounds has been restricted due to theirs undesired effects [5,6]. Because of the restrictions, an interest has been given to the antioxidants from natural sources. It was well known that plant constituents have antioxidant activity and free radicals scavenging effects. So, there is an increasing demand in safer and natural antioxidants for food, biological and pharmaceutical systems. Also, there are increasing trends in consumer preference towards natural and safer antioxidants from plants origin [7].

3. Present Study

In the study, we first determined the antioxidant activities of AEF and EEF using different antioxidant activities including DPPH, DMPD, ABTS and $O_2^{\bullet-}$ scavenging effects, total antioxidant activity-ferric thiocyanate method (FTC), Cu^{2+} and Fe^{3+} reducing abilities and metal chelating activity. Also, a significant purpose of this study was to show the main polyphenols in AEF and EEF by LC-MS/MS analysis.

Flaxseed was lyophilized as described previously [8]. Also, evaporated ethanolic extraction of flaxseed was obtained according to the previous method [5]. Total phenolics in AEF and EEF were measured by Folin-Ciocalteu reagent [4-6] and calculated as μg of GA equivalents (GAE) per one mg of dried extract. Total flavonoids in AEF and EEF were estimated using the $AlCl_3$ [9]. They were calculated as μg of quercetin equivalents (QE) per one mg dried extract. LC-MS/MS technique was used for evaluation the quantitative content of phenolics in AEF and EEF [10]. Standard chromatogram and chromatogram of phenols in AEF and EEF by LC-MS/MS (mg/mL) was summarized in Figure 1.

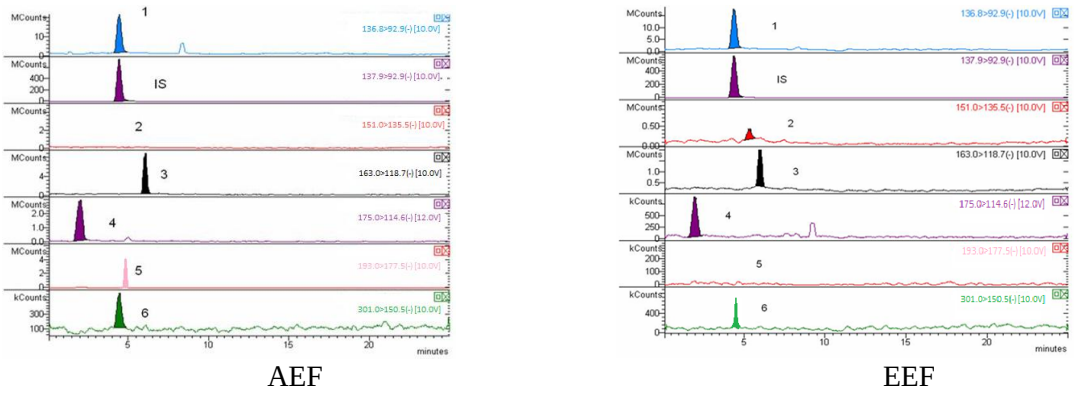

Figure 1. Chromatograms of phenolic antioxidants by LC-MS/MS

The experiments of validation and linearity of the used method for the indicated phenolics, the recovery of the experiments, the validation of parameters were determined for linearity, recovery, repeatability, limits of the detection (LOD), and quantification (LOQ) experiments, procedures of uncertainty were given previously [11]. Total antioxidant activity of AEF and EEF was performed according to FTC. Fe^{3+} reducing power was measured by reduction of Fe^{3+} [12] and Fe^{3+}-TPTZ complexes as described previously [13]. Cu^{2+} reducing capacity was done according to Apak et al. [14]. Metal chelating capacity was determined by disrupting of Fe^{2+}-ferrozine complex formation. Antiradical effects of AEF and EEF were determined by using the $DPPH^{\bullet}$, $DMPD^{\bullet+}$, $O_2^{\bullet-}$, and $ABTS^{\bullet+}$ scavenging [15]. The percentage of inhibition of lipid peroxidation, percentage of Fe^{2+} chelating and scavenging capabilities of radicals were calculated using the equation of A (%) = (1-λs/λc)x100 [16]. Where in A is the scavenged or chelated effect. λ_C and λ_S are absorbances of control and sample, respectively [17]. IC_{50} values indicate of the sample concentration, which was a required half concentration of sample for scavenging or chelating [18].

Plants have been used for treatment of many diseases [19]. Phenolic compounds are the most widely occurring chemicals, which having strong antioxidant properties [20]. The phenolic contents

(mg/g) in AEF and EEF were determined using the formula of y = 0.001x, (r^2:0.970). They were given as gallic acid equivalents (GAE) and found as 45.0 GAE/g for AEF and 167.0 GAE/g EEF. Phenolic compounds exhibit their antioxidant activity due to their redox property. According to the results obtained from LC-MS/MS analysis, the main phenolic acids in AEF and EEF were found to be p-hydroxybenzoic acid, ellagic acid, p-coumaric acid, ferulic acid and ascorbic acid, respectively. Ellagic acid (57 and 9 mg/kg), ellagic acid (85 and 13 mg/kg), p-coumaric acid (192 and 30 mg/kg) and p-hydroxybenzoic acid (779 and 120 mg/kg) were abundantly found in AEF and EEF, respectively (Table 1).

Recently, there is a growing interest in phenolic compounds, and flavonoids in particularly because of their antioxidant capacity and possible benefits in food and pharmaceutical applications and in human health [21]. The flavonoid contents (mg/g) in AEF and EEF was estimated using the following calibration curve (y:0.0085x, r^2:0.952). The total flavonoids in AEF and EEF were determined as 23.30 and 3.88 QE/g, respectively.

Antioxidants can easily reduce $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$ [22]. AEF and EEF had effective reducing in $Fe[(CN)_6]_3$ reduction method. As can seen in Table 2, AEF (r^2:0.940) and EEF (r^2:0.983) demonstrated effective Fe^{3+} reducing ability ($p<0.01$) and increased steadily with increased concentrations. The reducing power of AEF, EEF and standard compounds were found as follows: BHA (1.811, r^2:0.973), BHT (1.300, r^2:0.965) > Trolox (1.034, r^2:0.989) > EEF (0.340, r^2:0.983) > α-Tocopherol (0.240, r^2:0.935) > AEF (0.198, r^2:0.940). Reduction $Fe[(CN)_6]_3$ by antioxidants gives the intensive Perl's Prussian blue complex [23].

Cu^{2+}-neocuproine reducing power of AEF, EEF and standards (30 µg/mL) was shown in Table 2: BHT (0.828, r^2:0.981) > BHA (0.619, r^2:0.960) ≈ trolox (0.618, r^2:0.996) > EEF (0.437, r^2:0.952) ≈ α-Tocopherol (0.433, r^2:0.909) > AEF (0.172, r^2:0.938). Copper is a vital component for several endogenous antioxidant enzymes. It is known that well-regulated copper levels in diet had positive effects on cancer [24].

Table 1. LC-MS/MS results of selected phenolics and quantity of antioxidants in AEF and EEF (in mg/kg, AEF: aqueous extract of flaxseed shell, EEF: ethanol extract of flaxseed shell)

No	Compounds	Parent Ion	Daughter Ion	Collision Energy (V)	Amount of antioxidants (mg/kg)§	
					AEF	EEF
1	*p*-Hydroxybenzoic acid	136.8	92.9	10	779	120
2	Vanillin	151.0	135.5	10	-	8
3	*p*-Coumaric acid	163.0	118.7	10	192	30
4	Ascorbic acid	175.0	114.6	12	57	9
5	Ferulic acid	193.0	177.5	10	71	-
6	Ellagic acid	301.0	150.5	10	85	13
IS*	^{13}Cp-Hydroxybenzoic acid	137.9	92.9	10	-	-

Table 2. Antioxidant activity of AEF, EEF and standards at the same concentration (30 µg/mL; AEF: aqueous extract of flaxseed shell, EEF: ethanol extract of flaxseed shell, BHT: butylated hydroxytoluene, BHA: butylated hydroxyanisole)

	Total antioxidant activity	Fe^{3+} reducing ability*		Cu^{2+} reducing ability		FRAP Reducing assay	
	λ_{500}	λ_{450}	r^2	λ_{450}	r^2	λ_{593}	r^2
BHA	89.90	1.811	0.973	0.619	0.960	2.272	0.983
BHT	94.90	1.300	0.965	0.828	0.981	1.049	0.959
Trolox	93.10	1.034	0.989	0.618	0.996	1.672	0.995
α-Tocopherol	52.51	0.240	0.835	0.433	0.995	0.433	0.909
AEF	73.95	0.198	0.940	0.172	0.938	0.489	0.941
EEF	87.23	0.340	0.983	0.437	0.952	0.525	0.953

The FRAP assay measures the antioxidants ability to reduce $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ complex [24]. The most effective $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ reducing power was measured in BHA (2.272, r^2:0.983). This activity was greater than Trolox (1.672, r^2:0.995) > BHT (1.049, r^2:0.959) > EEF (0.525, r^2:0.953) ≈ AEF (0.489, r^2:0.941) > α-Tocopherol (0.433, r^2:0.959, Table 2). Also, a similar circumstance was

observed for the antioxidant capacity of AEF and EEF measured by either ABTS, CUPRAC or FRAP assays.

As seen in Table 3, AEF and EEF had a potent Fe^{2+} chelating effect ($p<0.01$). IC_{50} values for the Fe^{2+} chelating capacities of AEF and EEF were found as 9.24 µg/mL (r^2: 0.931) and 8.88 µg/mL (r^2:0.917). On the other hand, BHA had IC_{50} value of 24.75 µg/mL (r^2:0.995), BHT had IC_{50} value of 8.56 µg/mL (r^2:0.821), α-tocopherol had IC_{50} value of 17.76 µg/mL (r^2:0.939) and Trolox had IC_{50} value of 7.07 µg/mL (r^2:0.840). These results obtained from this test clearly showed that Fe^{2+} binding effect of AEF and EEF was similar to BHT and Trolox, but higher than that of BHA and α-tocopherol.

DPPH·, $DMPD^{\bullet+}$ and $ABTS^{\bullet+}$ reacts with an antioxidant compounds and can easily give hydrogen atoms [24]. The results showed that AEF and EEF showed effective radical scavenging effects when compared to BHT, BHA, Trolox and α-Tocopherol (Table 3). IC_{50} values of DPPH· scavenging for AEF, EEF, and standards on the DPPH radical were found as 53.30 µg/mL (r^2:0.934) for AEF, 49.50 µg/mL (r^2:0.966) for EEF, 6.42 µg/mL (r^2:0.778) for BHA, 38.50 µg/mL (r^2:0.843) for BHT, 5.87 µg/mL (r^2:0.932) for α-Tocopherol and 34.65 µg/mL (r^2:0.922) for Trolox (Table 3). Second improved radical scavenging technique is $ABTS^{\bullet+}$ scavenging activity. IC_{50} values for AEF and EEF in this assay were 27.72 µg/mL (r^2:0.945) and 25.56 µg/mL (r^2:0.962). Also, IC_{50} values belongs to $DMPD^{\bullet+}$ scavenging for BHA, BHT, α-Tocopherol and Trolox were found as 9.90 µg/mL (r^2:0.881), 9.45 µg/mL (r^2:0.842), 33.00 µg/mL (r^2:0.989) and 49.50 µg/mL (r^2:0.919), respectively. DMPD can form a steady and colored $DMPD^{\bullet+}$ cation. $DMPD^{\bullet+}$ had a maximum absorbance at 505 nm. IC_{50} for AEF and EEF was found as 28.88 µg/mL (r^2:0.924) and 27.72 µg/mL (r^2:0.914), respectively. IC_{50} was found as 22.35 µg/mL for BHA (r^2:0.793) and 21.00 µg/mL for Trolox (r^2:0.711).

Table 3. IC_{50} values of $DPPH^{\bullet}$ scavenging, $DMPD^{\bullet+}$ scavenging, $ABTS^{\bullet+}$ scavenging and $O_2^{\bullet-}$ scavenging activities and Fe^{2+} binding effects of AEF, EEF and standards

Compounds	DPPH• scavenging		$ABTS^{\bullet+}$ scavenging		$DMPD^{\bullet+}$ scavenging		$O_2^{\bullet-}$ scavenging		Fe^{2+} chelating	
	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2	IC_{50}	r^2
BHA	6.42	0.778	9.90	0.881	22.35	0.793	13.59	0.762	24.75	0.933
BHT	38.50	0.843	9.49	0.842	- **	- **	23.10	0.830	8.56	0.821
α-Tocopherol	5.87	0.932	33.00	0.989	- **	- **	26.65	0.855	17.76	0.939
Trolox	34.65	0.922	49.50	0.919	21.00	0.964	19.80	0.960	7.07	0.840
AEF	53.30	0.934	27.72	0.945	28.88	0.924	49.50	0.969	9.24	0.931
EEF	49.50	0.966	25.67	0.962	24.75	0.914	24.75	0.978	8.88	0.917

As can seen Table 3, IC_{50} values of scavenging of $O_2^{\bullet-}$ generation by same concentration of AEF and EEF and the other molecules were given. AEF and EEF were powerful inhibiting effects on $O_2^{\bullet-}$generation. IC_{50} value of $O_2^{\bullet-}$ scavenging effects of AEF and EEF were found to be 49.50 (r^2:0.969) µg/mL and 24.75 (r^2:0.978) µg/mL (Table 3). These values were calculated as 13.59 µg/mL (r^2:0.978) for BHA, 23.10 µg/mL (r^2:0.978) for BHT, 26.65 µg/mL (r^2:0.978) for α-Tocopherol and 19.80 µg/mL (r^2:0.978) for Trolox. It is obvious from these results $O_2^{\bullet-}$ scavenging activities of AEF and EEF are close standard compounds.

In conclusion, AEF and EEF were found as important antioxidant source in different *in vitro* bioanalytical tests like reducing abilities, total antioxidant activity, Fe^{2+} binding activities, and radical scavenging activities. Also, quantities of some phenolics including *p*-hydroxybenzoic, vanillin, *p*-coumaric acid, ascorbic acid, ferulic acid, and ellagic acid were successfully characterized by LC-MS/MS and *p*-hydroxybenzoic acid was the most abundant phenolic compound in both AEF and EEF. Also, this plant has been cultivated for fiber as well as a functional food because of its beneficial chemical composition, such as, tocopherols oils, fats enriched in ω3 fatty acids, crude fiber, protein and antioxidants in its seed. These components are probably also responsible for its antioxidant activity [25].

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