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Original Research Article

Extraction, purification, characterization and antioxidant activities of heat-resistance phenolic compounds from lemon pulp

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ABSTRACT

Lemon as a citrus species is widely cultivated in the Mediterranean region, Turkey. The lemon pulp has been characterized as a rich source of phenolic and flavonoid compounds, phenolic acid as well as novel coumarin derivatives. It is usually obtained within the juice production in the food industries. In the current report, lemon pulp was initially dried and immediately after subjected to extraction with hot water and ethyl acetate. This study deals with the isolation of secondary metabolites along with the determination of 32 phenolic contents. After isolation of major components using Sephadex LH-20 column and silica gel, 11 phenolic components were totally characterized using the 1D and 2D NMR techniques. Lemon pulp known as citrus peel has been recognized as a potential source of coumarin, limonoids, and methoxylated flavones. The antioxidant activities of the limonin (LMN) and lemon pulp ethyl acetate extract (LPEA) were evaluated using *in vitro* methods involving total antioxidant activity, reducing power, inhibition of lipid peroxidation, scavenging activities of $DPPH^{\cdot}$, H_2O_2 and $ABTS^{+\cdot}$ and compared with standards at 10, 25, 50 and 100 $\mu g.mL^{-1}$. According to the finding of this research, LMN and LPEA showed strong *in vitro* antioxidant activities and also significantly exhibited an increase to be in a dose-dependent manner ($p<0.05$).

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1. Introduction

Lemon pulp is practically produced during the preparation of lemon. The pulp generally includes dissolved materials such as seed and lemon peel. Lemon peel and seed contain high contents of phenolic components such as flavonoids, coumarone, phenolic acid, and phenols. Flavonoids are considered as important and main secondary metabolites of plants. To date, more than 8000 compounds belonging to flavonoids have been identified which are linked to various methoxyl, hydroxyl, and glycoside groups (Erenler et al., 2016). Citrus fruits are one of the most important sources of flavonoids of which lemon peel is a rich source of hesperidin, eriodictiol, diosmin and their glycoside derivatives (Miyake et al., 1997a). Eridictiol, hesperidin and different glycosides derivatives are so prevalent in lemon peel. Moreover, lemon peel contains neoeriocitrin, naringin and neohesperidin, whereas the lemon seed has considerable amounts of hesperidin and eriocitrin together with their derivatives. According to the literature, these compounds possess high antioxidant activities (Bocco et al., 1998; Miyake et al., 1998). Eriocitrin exhibited the greatest antioxidant activity compared with the other citrus flavanoids (Miyake et al., 1997b). Eriocitrin, hesperidin and diosmin are among the most widespread flavonoid glycosides in the citrus species (Del Rıo et al., 2004). Polymethoxy flavonoids are mostly found in citrus species such as sinensetin, one of the most abundantly polimethoxy flavones in mandarin peel (Nogata et al., 2003). The flavanones (hesperidin, neohesperidin, narirutin, naringin) and polymethoxylated flavones (sinensetin, nobiletin, tangeretin) mostly include citrus species (M'hiri et al., 2017).

According to the literature, most flavonoids posed

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antiproliferative activity (Gecibesler et al., 2017; Yildiz et al., 2017). One of the previous reports demonstrated that citrus flavonoids opposed the human breast cancer cell line (So et al., 1996). This study accounted for the efficiency of citrus flavonoids against the *in vitro* human breast cancer cell line. Naringenin is a citrus flavanoids and has been characterized to have potential anticancer activity against the the colon cancer cell line (Erlund et al., 2001). Lemon pulp contains remarkable quantities of flavanoids and particularly phenolic acids. Clorogenic acid, p-coumaric acid, sinapic acid and caffeic acid are main phenolic acid components of the lemon peel, as well (Wang et al., 2008). Coumarin derivatives are the other important components of lemon peel. Coumarin is highly concentrated in the peel oil of lemon. In a related study, a chemical compound, namely 5-geranyloxy-7-methoxy coumarone which was found to be active against the oral bacteria was successfully isolated and characterized (Miyake and Hiramitsu, 2011). In addition, citrus coumarins, namely umbelliferen and auraptene were separated from natsumican as one of the citrus species. These compounds have shown promising inhibitory activities against the mice cancer cell line (Murakami et al., 1997; Mazimba, 2017). Limonin and nomilin are major components which lactones of the lemon peel oil and these compounds posed strong antioxidant activities (Sun et al., 2005).

Although many researches have focused on the lemon residue, information on phenolics and isolation of secondary metabolites of lemon pulp is lacking. Therefore, in the present study, our main goal was to investigate the antioxidant impact of water-soluble ethyl acetate extracts in order to isolate the natural constituents from lemon pulp and quantify the total phenolic contents.

2. Experimental

2.1. Samples and chemicals

Lemon pulp supplied from DIMES juice factory on 17 September, 2016 and dried at room temperature in sunless storage for one week. In this preliminary step, 5 kg of dry pulp obtained. The HPLC grade solvents including ethyl acetate, methanol, chloroform, hexane, *n*-butanol were purchased from Merck. Sephadex-LH 20 and silica gel was purchased from Sigma-Aldrich.

2.2. Extraction procedure

The waste lemon pulp was supplied from the fruit juice factory (DİMES juice factory, Tokat) collected from lemon trees (*Citrus lemon* L.) (Fig. 1). Firstly, it was dried in the shadow. The lemon pulp, which had a dry weight of about 5 kg, was boiled in reflux system for 2 hours with distilled water and subjected to aqueous extraction as explained in the literature except using specially designed distillation apparatus as seen in Fig. 2 (Demirtas et al., 2013). The aqueous extract was separated by filtration and extracted with ethyl acetate. This process was continued until significant amounts of the aqueous components were transferred to ethyl acetate. When this process was carried out three times, the extraction was completed with ethyl acetate. The remaining aqueous extract was subjected to extraction in the same manner as *n*-butanol. The obtained ethyl acetate and *n*-butanol extracts were separated from the solvent by rotary evaporator to give a dry crude extract. Eventually, 13.2 g of ethyl acetate and 21 g of *n*-butanol crude extracts were obtained from the lemon pulp.

Fig. 1. Lemon trees at early period (left) and at late period (right) show the seasonal differences.

Fig. 2. The distillation apparatus was designed by Ayhan AKPEK for boiling fruit pulps (pictured by Mehmet Ali DEMİRCİ at Çankırı Karatekin University).

2.3. Isolation of phenolic contents

The crude ethyl acetate extract (EA) was resolved with perpetrated methanol/ethyl acetate/hexane mix solvent system and applied to Sephadex LH-20 column. The EA (13.2 g) was separated by column chromatography (CC) and eluted with methanol/ethyl acetate/hexane (5:5:1). The fractions were collected to sterile tubes. The fractions were monitored using TLC and viewed under UV light (254 and 365 nm) or by spraying with seric sulfate reagent followed by heating at 100 °C. Similar fractions were combined by TLC monitoring to obtain 7 fractions (A-G). Fraction A was purified by recrystallization to give compound **10** as

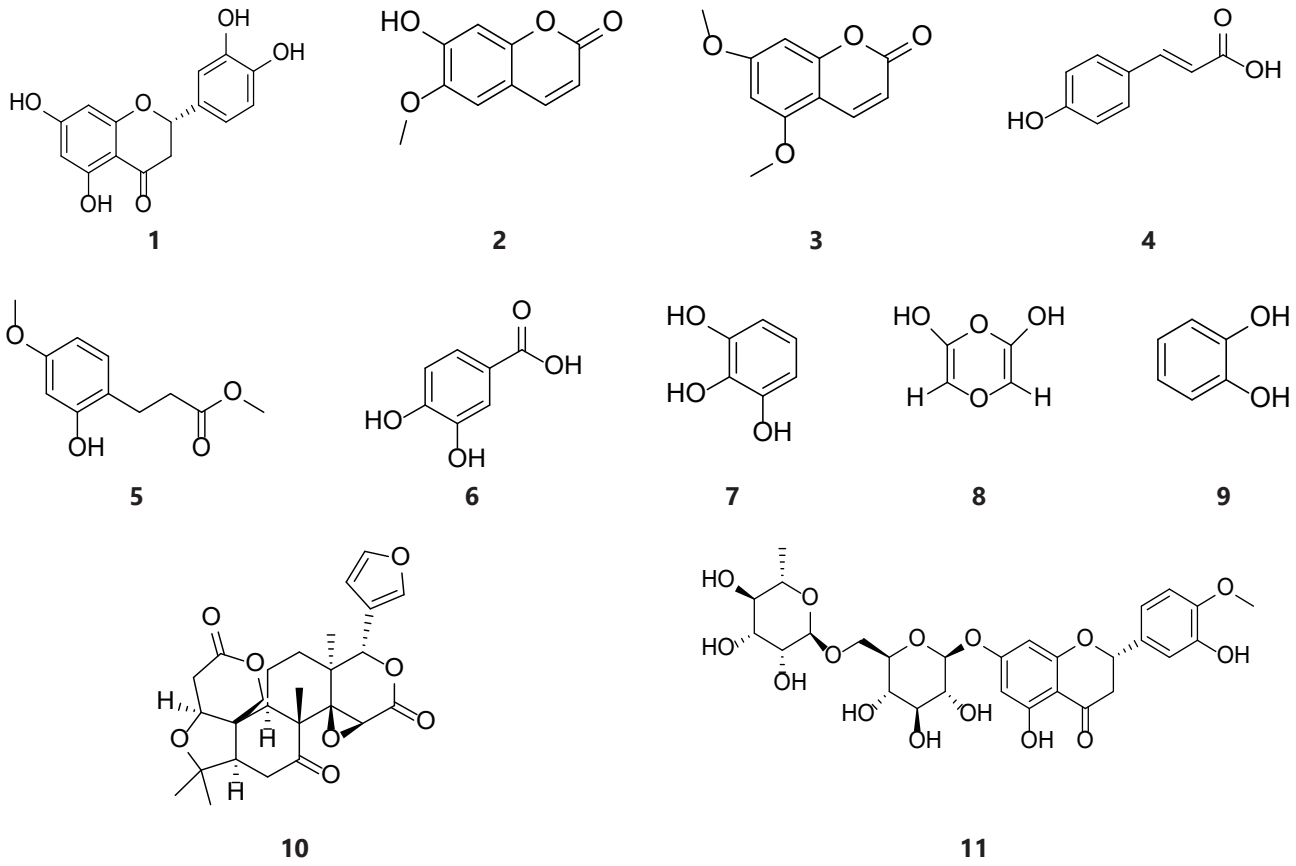

Fig. 3. The structures of compounds isolated from lemon pulb. Eriodictyol (**1**), scopoletin (**2**), citropten (**3**), *p*-coumaric acid (**4**), 2-hydroxy-4-methoxy benzenepropanoic acid methyl ester (**5**), protocatechuic acid (**6**), pyrogallol (**7**), diglycolic anhydride (**8**), catechol (**9**), limonin (**10**), hesperidin (**11**).

white crystals.

Fraction B was further separated by CC and eluted with ether/methanol/hexane (EMH, 10:10:1) to give four subfractions (B1-B4). Subfraction B3 was then separated by CC (EMH) to obtain compound **2** from fraction B3/7 and repeated fractionations of B2/3 by silica gel to give compounds **5** and **3** from fractions B2/3-1a and B2/3-1b, respectively.

Fraction C was separated by CC using Sephadex LH-20 and eluted with EMH to obtain compounds **4**, **9** and **11** from fractions C4/1, C4/2 and C4/10, respectively.

Fraction D was separated by CC using EMH solvent mixture to give compound **6** from fraction D2/6 and subfraction (D3) was subjected to silica gel CC with gradient eluents of chloroform and methanol which finally afforded compounds **7**, **8** and **1** from fractions D3/2-1, D3/2-2 and D3/4-1, respectively. The structures of all compounds are given in Fig. 3.

2.4. Lemon pulp characterization

The resulting water-soluble lemon pulp was chemically characterized in the same way as the solvent extracts of plants. In addition, the chemical contents of the ethyl acetate extract obtained from the water-soluble part of the lemon pulp were determined using HPLC-TOF/MS equipment with its library search.

2.5. HPLC-TOF/MS analysis

Phenolic compounds of the extracts were quantified by using Agilent 1260 Infinity HPLC system (Agilent, Santa Clara, CA) coupled with an Agilent 6210 TOF-MS detector and an Agilent Zorbax SB-C_{18} column (100 mm 4.6 mm 3.5 µm). Fig. 4 shows the HPLC-TOF/MS chromatograms of the isolated and quantified components obtained from crude LPEA extract. The dried crude LPEA extract (100 µg) was dissolved in DMSO at room temperature. The sample was filtered through a PTFE (0.45 µm) filter by an injector to remove particulates. The analysis of phenolic compounds was carried out as described by Demirtas et al. (2013). The compounds were identified tentatively by comparing the mass spectra with MassHunter METLIN PCDL (Agilent, 24768 compounds) and publicly available databases for phenolic compounds. Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile, respectively. The flow-rate was 0.8 mL min^{-1}, column temperature was 35 ºC, and the injection volume was 5 µL. The elution program was as follows: 0-1 min, 10% B; 1-12 min, 40% B; 12-14 min, 90% B; 14-17 min, 90% B; 17-18 min, 10% B; 18-25 min, 10% B. Ionization mode of

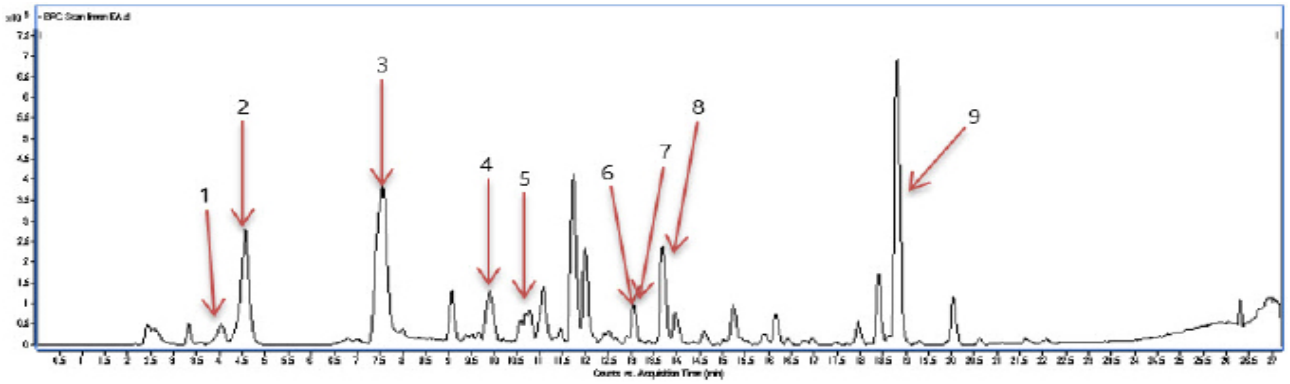

Fig. 4. HPLC-TOF/MS chromatogram for isolated compounds of pyrogallol (**1**), protocatechuic acid (**2**), catechol (**3**), *p*-coumaric acid (**4**), hesperidin (**5**), scopoletin (**6**), citropten (**7**), eriodictyol (**8**), limonin (**9**).

HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 ºC, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. Phenolic compounds were identified by the retention time of sample chromatographic peaks being compared with those of authentic standards using the same HPLC operating condition, high resolution mass and also by the extract being spiked with the standard components.

2.6. Biological activities

2.6.1. Total antioxidant by phosphomolybdenum method

The total antioxidant activity of the sample was evaluated following the method given in literature (Prieto et al., 1999) based on the reduction of Mo(VI) to Mo(V) by antioxidant and the subsequent formation of specific green phosphate/Mo(V) compounds. BHT, TBHQ and trolox were used as positive controls.

2.6.2. Assay of free radical scavenging

The electron donation activity of the sample was measured spectrophotometrically by bleaching of the purple-colored solution of free radical scavenging activity according to the technique available in literature with a slight modification (Blois, 1958). BHT, TBHQ and trolox were used as positive controls.

2.6.3. $ABTS^{+\cdot}$ scavenging assay

The $ABTS^{\cdot+}$ scavenging activity was evaluated spectrophotometrically according to the previously described by assay with slight modifications (Ozen et al., 2017). BHT and α-tocopherol were used as positive controls.

2.6.4. Assay of reducing power

In this test, the reductant samples cause the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form and monitored by measuring the absorbance at 700 nm (Oyaizu, 1986). BHT, TBHQ and trolox were used as positive controls.

2.6.5. Assay of metal chelating

The metal chelating activity of the sample was assessed as described by literature (Dinis et al., 1994) using EDTA as a positive control.

2.6.6. Assay of H_2O_2 scavenging

The H_2O_2 scavenging activity of the sample was measured according to the slightly modified method given by the reference (Zhao et al., 2006). BHT, TBHQ and trolox were used as positive controls.

2.6.7. Inhibition of linoleic acid peroxidation

The inhibition of peroxidation assays of the sample was tested using the inhibition of linoleic acid peroxidation according to the slightly modified method given in the literature (Choi et al., 2002). BHT, TBHQ and trolox were used as positive controls.

2.7. Statistical analysis

The results were represented as mean values and standard deviations (mean ± SD). Statistical comparison of means between the samples were analyzed with one-way analysis of variance (ANOVA) and Tukey's using SPSS 20.0, respectively. The statistical differences of results were considered to be significant ($p<0.05$). All data were performed in triplicates.

3. Results and Discussion

3.1. Extraction of polyphenols

The air-dried lemon pulp was extracted with boiling water before extraction with organic solvents as the essential part of the isolation of phenolic contents, due to the solvent-free residue of the lemon pulp for

different purposes. The leftover lemon juice pulps can be obtained from one of the juice factories in Turkey more than 20 tons a year. The water-soluble part of the aqueous lemon pulp extracts can represent an important source of raw material for the production of value-added products such as phenolics and flavonoids for different applications.

3.2. Characterization of the isolated compounds

Eleven phenolic compounds were isolated for the first time with a different method from the extract which was obtained from the water-soluble part of the leftover lemon pulp. The structures of the isolated compounds **1**-**11** (Fig. 3) were identified as eriodictyol (**1**), scopoletin (**2**), citropten (**3**), *p*-coumaric acid (**4**), 2-hydroxy-4-methoxy benzenepropanoic acid methyl ester (**5**), protocatechuic acid (**6**), pyrogallol (**7**), diglycolic anhydride (**8**), catechol (**9**), limonin (**10**), hesperidin (**11**) on the basis of HPLC-TOF/MS, NMR analysis and also confirmed by comparison with data in the literature (Miyake et al., 1997a, b; Miyake et al., 1998).

Isolation and characterization techniques of the bioactive compounds are considered as the most important steps in the practical application of the polyphenols. The current paper deals with the separation and characterization methods, the main advantages as well as the limitation of sephadex based chromatographic method to establish the most feasible methods for the analysis of polyphenols.

According to the retention time of calibration standards, LPEA presented a chemical profile composed of fourteen phenolic acids and eighteen phenolics and flavonoids belonging to different classes. Gentisic, *p*-coumaric and 4-hydroxy benzoic acids can be considered as the major phenolic acids. As seen in Table 1, hesperidin and neohesperidin were determined as the major flavonon glycosides. Moreover, hesperidin was isolated using the aforementioned chromatographic methods, as well (Fig. 3).

Table 1
Quantification of phenolic compounds of LPEA.

Phenolics/flavonoids	LPEA (mg kg^{-1})
Gentisic acid	127.11
Chlorogenic acid	tr[a]
4-Hydroxybenzoic acid	11.15
Protocatechuic acid (**6**)	3.56
Caffeic acid	2.74
Vanillic acid	8.13
Syringic acid	1.20
Rutin	1.73
Polydatin	3.33
Scutellarin	tr
Quercetin-3-ß-D-glucoside	0.32
Naringin	1.80
Diosmin	6.25
Taxifolin	1.15
Hesperidin (**11**)	17.79
Neohesperidin	11.02
Morin	1.67
Salycilic acid	tr
Quercetin	0.18
Sinnamic acid	0.58
Naringenin	tr
Kaempferol	tr
Gallic acid	2.66
Fumaric acid	3.51
Sinapic acid	9.32
p-Coumaric acid (**4**)	59.78
Catechin	tr
4-Hydroxybenzaldehyde	tr
Ferulic acid	7.48
Apigenin	0.75
Diosmetin	tr
Eupatorin	tr

[a]tr: trace

3.3. Evaluation of antioxidant activities

Evaluation of antioxidant activity was conducted by seven methods involving total antioxidant activity, reducing power, DPPH$^{\cdot}$ scavenging activity, ABTS$^{+\cdot}$ scavenging activity, metal chelating activity, H_2O_2 scavenging activity and inhibition of lipid peroxidation) for limonin (LMN) and lemon pulp boiled with distilled water and extracted with ethyl acetate (LPEA) as compared to BHT (butylated hydroxytoluene), TBHQ (t-butyl-hydroxyquinone), trolox, α-tocopherol and EDTA (Table 2).

3.4. Total antioxidant activity

The sample of total antioxidant capacity was evaluated by the phosphomolybdenum method for the spectrophotometric quantitative determination. This test is based on reducing from Mo(VI) to Mo(V) by the sample and then forming a green phosphate/Mo(V) complex at an acidic pH. The phosphomolybdenum method was characterized and optimized for molar absorption coefficient for linearity, reproducibility and several standard antioxidants (Ozen et al., 2017). A remarkable increase of the total antioxidant activity with tested LMN, LPEA and standards were exhibited with increasing their concentrations, significantly (p<0.05). The formation of phosphate-Mo(V) complex was found to increase in the order of LMN>TBHQ>BHT>LPEA>trolox with 972, 894, 671, 642, and 466 µmol α-tocopherol equivalent/g at 100 µg.mL^{-1}, respectively (Table 2). The doses of LMN were higher than that of LPEA, BHT, TBHQ and trolox at 10, 25 and 50 µg.mL^{-1}, significantly (Table 1).

Table 2

Total antioxidant activity, inhibition of linoleic peroxidation, reducing power, free radical scavenging, $ABTS^{+\cdot}$ scavenging, hydrogen peroxide scavenging and metal chelating activity of the LMN, LPEA and standards at 10-100 µg.mL^{-1}. The values were represented means ± SD (n=3). The data were evaluated using superscript letters (a-d) and were significantly different at $p<0.05$.

Samples[a]	*In vitro* antioxidant activities			
	10 µg.mL^{-1}	25 µg.mL^{-1}	50 µg.mL^{-1}	100 µg.mL^{-1}
Total antioxidant activity, µmol α-tocopherol equivalent/g				
LMN	48.21 ± 8.61^{a}	131.68 ± 9.81^{b}	305.63 ± 5.22^{c}	972.58 ± 15.76^{d}
LPEA	36.16 ± 1.11^{a}	91.09 ± 5.46^{b}	214.76 ± 6.76^{c}	642.83 ± 6.64^{d}
BHT	16.37 ± 5.54^{a}	44.85 ± 2.51^{a}	215.59 ± 3.84^{b}	671.85 ± 11.09^{c}
TBHQ	9.15 ± 0.72^{a}	53.94 ± 2.42^{b}	228.98 ± 4.64^{c}	894.38 ± 12.35^{d}
Trolox	6.10 ± 0.43^{a}	38.06 ± 2.66^{b}	122.70 ± 3.85^{c}	466.80 ± 5.56^{d}
Reducing power, 700 nm				
LMN	0.098 ± 0.024^{a}	0.102 ± 0.017^{a}	0.106 ± 0.015^{a}	0.113 ± 0.007^{a}
LPEA	0.128 ± 0.036^{a}	0.164 ± 0.002^{a}	0.260 ± 0.006^{b}	0.409 ± 0.004^{c}
BHT	0.201 ± 0.014^{a}	0.333 ± 0.013^{ab}	0.429 ± 0.015^{b}	0.761 ± 0.050^{c}
TBHQ	0.165 ± 0.016^{a}	0.275 ± 0.007^{b}	0.354 ± 0.021^{c}	0.630 ± 0.023^{d}
Trolox	0.146 ± 0.005^{a}	0.203 ± 0.001^{a}	0.265 ± 0.029^{b}	0.485 ± 0.034^{c}
Free radical scavenging activity ($DPPH^{\cdot}$), %				
LMN	45.27 ± 8.81^{a}	49.49 ± 2.06^{ab}	55.65 ± 4.07^{bc}	60.76 ± 1.27^{c}
LPEA	13.82 ± 7.29^{a}	38.64 ± 5.90^{b}	80.07 ± 1.42^{c}	85.82 ± 5.88^{c}
BHT	8.67 ± 1.73^{a}	16.02 ± 1.50^{a}	25.00 ± 1.65^{b}	61.88 ± 6.02^{c}
TBHQ	87.39 ± 6.01^{a}	94.66 ± 0.38^{ab}	95.06 ± 0.40^{bc}	95.75 ± 0.33^{c}
Trolox	92.30 ± 1.20^{a}	94.03 ± 1.49^{ab}	95.24 ± 0.67^{c}	97.59 ± 0.60^{c}
$ABTS^{+\cdot}$ scavenging activity, %				
LMN	2.13 ± 0.11^{a}	9.69 ± 0.66^{b}	13.81 ± 1.10^{c}	15.93 ± 1.21^{c}
LPEA	6.09 ± 1.28^{a}	14.38 ± 1.27^{a}	32.72 ± 0.94^{b}	64.64 ± 4.31^{c}
BHT	2.99 ± 0.81^{a}	6.41 ± 0.79^{b}	12.61 ± 0.73^{c}	18.09 ± 0.84^{d}
α-tocopherol	3.70 ± 0.41^{a}	4.77 ± 0.31^{a}	9.97 ± 1.19^{a}	20.52 ± 5.88^{b}
Hydrogen peroxide scavenging activity, %				
LMN	21.55 ± 3.66^{a}	32.76 ± 9.75^{ab}	53.45 ± 9.75^{bc}	59.48 ± 8.53^{d}
LPEA	53.57 ± 5.79^{a}	61.90 ± 6.73^{a}	64.29 ± 3.37^{a}	66.67 ± 6.73^{a}
BHT	9.09 ± 5.43^{a}	27.27 ± 1.29^{ab}	43.18 ± 5.79^{bc}	48.64 ± 4.50^{d}
TBHQ	15.45 ± 2.57^{a}	20.45 ± 9.64^{a}	39.55 ± 8.36^{a}	27.73 ± 3.21^{a}
Trolox	9.55 ± 1.93^{a}	29.55 ± 9.64^{ab}	41.82 ± 2.57^{b}	39.55 ± 5.79^{b}
Metal chelating activity, %				
LMN	18.04 ± 1.75^{a}	26.34 ± 1.66^{b}	31.08 ± 1.82^{bc}	32.77 ± 3.21^{c}
LPEA	23.07 ± 3.04^{a}	30.65 ± 3.89^{b}	31.20 ± 2.59^{b}	43.90 ± 1.39^{c}
EDTA	45.17 ± 2.67^{a}	64.50 ± 4.56^{b}	97.43 ± 2.08^{c}	98.10 ± 1.02^{c}
Inhibition of linoleic acid peroxidation, %				
LMN	20.94 ± 6.56^{a}	66.51 ± 2.76^{b}	66.98 ± 1.70^{b}	90.40 ± 1.09^{c}
LPEA	71.80 ± 0.40^{a}	62.72 ± 0.98^{b}	73.79 ± 2.15^{c}	77.35 ± 1.27^{c}
BHT	84.96 ± 1.27^{a}	83.36 ± 1.65^{a}	92.68 ± 1.68^{b}	94.70 ± 2.31^{b}
TBHQ	43.39 ± 6.44^{a}	52.26 ± 3.77^{a}	53.73 ± 6.90^{a}	68.82 ± 2.22^{b}
Trolox	3.80 ± 1.01^{a}	31.78 ± 7.08^{b}	54.41 ± 6.85^{c}	71.97 ± 8.40^{d}

[a]Abbreviations: LMN: limonin, LPEA: limon pulp ethyl acetate extract, BHT: butylated hydroxytoluene, TBHQ: t-butyl-hydroxyquinone

3.5. Reducing power

The reducing power ($Fe^{3+} \rightarrow Fe^{2+}$) is commonly used to determine the ability of a new antioxidant to donate an electron. In the test, the presence of a reductant results in the reduction of the Fe^{3+}-ferricyanide complex to the Fe^{2+}. Then, the amount of Perl's Prussian blue is monitored using a spectrophotometric method (Bursal and Köksal, 2011). Table 2 exhibits the reducing power of LMN, LPEA, and standards basing on the determination of the absorbance values at 700 nm. As their concentrations increased, the reducing capacity of samples were gradually enhanced and exhibited a dose-dependent reducing power, except for LMN. The reducing power of LMN was less than the same concentration of LPEA and standards, but LPEA had a significantly stronger reducing power than LMN at the same concentration ($p<0.05$) due to the frequency of different limonin derivatives in the ethyl acetate extract. These compounds containing the substitution of hydroxyl groups can contribute to the antioxidant activity of LPEA (Table 2).

3.6. Free radical scavenging activity

$DPPH^{\cdot}$, a synthetic nitrogen-centered radical, is widely used to evaluate the free radical scavenging activity of antioxidant samples. When the unpaired electron of the $DPPH^{\cdot}$ is mixed with an antioxidant at 517 nm, the antioxidant agent donates an electron to the $DPPH^{\cdot}$, and also purple color changes to the yellow color (Ozen et al., 2017). The free radical scavenging activities of the studied LMN, LPEA, BHT, TBHQ and trolox were observed to exhange with increasing their concentrations. Table 2 shows the $DPPH^{\cdot}$ scavenging activities of the samples. LMN exhibited a higher free radical scavenging activity than BHT at 10, 25 and 50 µg.mL^{-1} concentrations. However, the highest scavenging activity was recorded for TBHQ and trolox a percentage value of 95 and 97% at a concentration of 100 µg.mL^{-1}, respectively (Table 2).

3.7. $ABTS^{+\cdot}$ scavenging activity

The $ABTS^{+\cdot}$ is generated by the oxidation of ABTS by $K_2S_2O_8$, and when an antioxidant is mixed, $ABTS^{+\cdot}$ is scavenged and converted to a nonradical molecule (Re et al., 1999). As shown in Table 2, LMN and LPEA were found to be considerable scavengers of the $ABTS^{+\cdot}$. Their scevenging activities were dose-dependent. From the highest to the lowest, the antioxidant activities of

LPEA, α-tocopherol, BHT, and LMN were determined as 64, 20, 18 and 15% at 100 µg.mL^{-1}, respectively. LMN exhibited higher ABTS$^{+\cdot}$ scavenging activity than α-tocopherol and BHT at 25 and 50 µg.mL^{-1} (Table 2).

3.8. Metal chelating activity

The transition metals play an important role, and also lead to the formation of $O_2^{\cdot-}$ and $^{\cdot}OH$ through an oxidative reaction (Haber and Weiss, 1934). These free radicals may accelerate the lipid peroxidation, protein, and DNA damage. In addition, the active transition metals transfer a single electron within the oxidation reactions. The antioxidants inhibit the oxidation by metal chelate activity, reduce redox potential and stabilize metal oxide. A new chelator structure is effective as natural products due to inhibiting the transition metal-dependent and stabilizing process of the oxidized form of the active metals. The LMN and LPEA interfered with the formation of Fe^{2+}-ferrozine complex before ferrozine and resulted in a decrease in the red-color of complex. Table 2 presents the Fe^{2+}-chelating activities of the LMN, LPEA and EDTA at various concentrations. The values for the Fe^{2+}-chelating activities of LMN, LPEA and EDTA were 32, 43 and 98% at 100 µg.mL^{-1}, respectively (Table 2). These results indicate that LMN and LPEA had considerable Fe^{2+}-chelating abilities.

3.9. Hydrogen peroxide scavenging activity

In biological systems, hydrogen peroxide forms *in vivo* by antioxidant enzymes and indicates a precursor to produce the $^{\cdot}OH$ radicals. The $^{\cdot}OH$ radicals are able to cause tissue damage, react with most biomolecules cell death and cross cell membrane (Poprac et al., 2017). Thus, scavenging of $^{\cdot}OH$ radicals is very important for the elimination of cell. Therefore, we aimed to evaluate the hydrogen peroxide scavenging activity of the new natural products. The percentage of hydrogen peroxide scavenging ability by LMN, LPEA and comparison with the effect of standards (BHT, TBHQ and trolox) at 10, 25, 50 and 100 µg.mL^{-1} have been tabulated in Table 2. Their activities were the dose-dependent manner in a test, significantly (p<0.05). The LMN and LPEA exhibited effective hydrogen peroxide scavenging activity higher than standards at the same doses.

3.10. Inhibition of linoleic peroxidation

The substrates resulting from oxidation along with the defense system mechanism, can be continuously initiated or promoted by different chemical and physical processes. Target substances include oxygen, polyunsaturated fatty acids, phospholipids, cholesterol and DNA (Antolovich et al., 2002). Lipid peroxidation, being a complex free radical chain process, involves an array of radicals and is measured by the amount of peroxide and the primary products of lipid oxidation produced during the initial stages of oxidation (Frankel, 2014). The LMN and LPEA affected the non-enzymatic linoleic peroxidation at the tested concentrations and inhibited the linoleic peroxidation induced by Fe^{2+} in a dose-dependent manner, significantly (p<0.05) (Table 2). The inhibition assays extent to LMN, LPEA and standards in the comparable levels at 10, 25, 50 and 100 µg.mL^{-1} (Table 2). The outcomes expressly showed that LMN and LPEA had a higher inhibition of linoleic peroxidation than that of TBHQ and trolox at high dose, but lower than BHT.

4. Concluding remarks

Due to the typical characteristics of a lemon pulp and taking into account the raw material to be used for its production, the present study revealed high phenolic contents of pulp for the production of natural antioxidants, as it has been concluded that these types of pulps have a promising future in the production of food additives. The findings of this work showed that both extracts and isolated compounds contain valuable biological active components, such as potent antioxidants. The gentisic acid (127.11 mg kg^{-1}) and *p*-coumaric acid (59.78 mg kg^{-1}) are the main aromatic organic acids among the all determined 32 components by HPLC-TOF/MS. In this sense, it is expectable that the obtained pulp from lemon residue presents interesting properties in terms of food reinforcement for natural antioxidants.

Finally, the findings of this study may help juice factories for evaluation of the pulps in a different way. In addition, this report may encourage business owners to produce valuable components from fruit pulps. However, extensive studies on other fruit pulps are required to provide further clues about their possible pharmaceutical and nutritional explorations for the human health.

Conflict of interest

The authors declare that there is no conflict of interest.

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