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[1]Laboratory of Methods and Analysis Techniques (LMTA), National Institute of Research and Physico-Chemical Analysis (INRAP), Sidi Thabet, Ariana, Tunisia
[2]Department of Ecology, Faculty of Sciences of Tunis, El Manar, Tunis, Tunisia

Maturational effects on phenolic constituents, antioxidant activities and LC-MS / MS profiles of lemon (*Citrus limon*) peels

Nadia Mcharek[1*], Belgacem Hanchi[1,2]

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Summary

Methanol extracts from peels of lemon (*Citrus limon* (L.) Burm) fruits collected at three maturity stages (immature, half-mature and mature) were analyzed for their total phenolic contents (TPC) and antioxidant activities. Irrespective to peel parts analyzed (albedo or flavedo), a declining trend was observed for TPC during maturation. By using DPPH, ABTS and FRAP assays, it was found that all extracts had strong antioxidant activities. As maturation progress, the antioxidant activity of both peel parts decreased. Significant relationships between antioxidant capacity and TPC were found, indicating on the one hand, that phenolic compounds are the major contributors to the antioxidant properties, and that the three methods have similar predictive capacity for antioxidant activity of lemon peel, on the other hand. Analysis by HPLC-DAD-MS/MS revealed that flavedo and albedo extracts haves similar phenolic profiles and allowed the identification of hesperidin, rutin, eriocitrin, diosmin and hyperoside, as the main compounds.

Introduction

Plants particularly those of the horticulture section are used by people for food, either as edible products or for culinary ingredients, medicinal products, and ornamental and aesthetic purposes. They represent a genetically very diverse group and play a major role in modern society end economy. Fruits and vegetables are an important component of traditional food, but possess also a central role in healthy diets of modern urban population (Kaczmarska et al., 2015; Mlcek et al., 2015; Wojnicka-Poltorak et al., 2015).
Citrus (*Rutaceae* family) plants are the most important fruit tree crop in the world, with an annual production of approximately 1.02 hundred million tons (Hwang et al., 2012). The fruits are the most popular for consumers throughout the world owing to their pleasant flavors and nutritional value (Qiao et al., 2008). They are mainly used for dessert, juice and jam production. They are also credited with a long list of medicinal uses including antioxidant, antimicrobial, antifungal, anti-inflammatory, antitumoral and hypoglycemic, among others (Di Vaio et al., 2010; Espina et al., 2011; Tripoli et al., 2007). These functional properties were attributed to a plethora of bioactive ingredients including vitamin C, dietary fibres, folic acid, essential oils and flavonoids (Senevirathne et al., 2009).
Within the genus *Citrus*, lemon (*C. limon*) has received particular attention and it is considered as a consolidated source of functional ingredients namely vitamin C, minerals, carotenoids, limonoids and flavonoids (Gonzalez-Molina et al., 2008). The latter components usually obtained from the peel, are of particular interest due to their intriguing biological properties, such as antioxidant, anti-inflammatory, anticancer, chemopreventive, cardioprotective and neuroprotective activities (Benavente-Garcia and Castillo, 2008; Hwang et al., 2012). Previous phytochemical studies have revealed that lemon flavonoids are mainly composed of flavanones, flavones, and polymethoxyflavones (Gil-Izquierdo et al., 2004). Hesperidin, disometin and luteolin, to which a single or multiple functional properties are ascribed, are reported as the main lemon flavonoids (Hwang et al., 2012). However, as commonly happens in many plant species, the phenolic constituents are particularly prone to quantitative and qualitative variations depending on genotype, plant part analyzed, environmental conditions, cultural practices and also on the stage of maturity of the fruit (Del Rio et al., 2004; Gonzalez-Molina et al., 2008; Kawaii et al., 1999) Changes occurring during the fruit maturation stages can affect the nutritional value and health properties of the lemon fruit; hence it is important to determine the optimum developmental stage having the maximum functional properties.
In the specific case of lemon, the effect of fruit maturation has received little attention, and the only report available showed that the maturation process was associated with deep quantitative changes in flavonoid constituents (Del Rio et al., 2004). However, the latter study was limited to the characterization and quantitation of these constituents without considering their biological activity, which was ultimately affected by the type and quantity of flavonoids. This knowledge is, however, crucial to define the possible application areas for the rational use of lemon peels.
In Tunisia, the area under citrus cultivation was estimated to be about 19.250 ha with an annual production of 230.000 tons, covering the fresh fruit market, agro-food industry and the exportation demands (Hosni et al., 2010). In Food and agro-food industries, considerable amounts of wastes or by-products such as peels, seeds and pulps are produced. These by-products, however, could represent an interesting source of dietary fibres, phenols and essential oils, among others (Garau et al., 2007). Consequently, there is a considerable emphasis on the recovery and upgrading to higher value and useful products of these by-products.
Despite their socio-economic relevance, comprehensive phyto-chemical investigations on phenolic constituents of Tunisian *Citrus* species are scanty. Bearing this in mind, the present study intends to: (a) investigate the evolution of total phenolic contents in relation to peel parts (flavedo and albedo) and during fruit maturation, (b) characterize the main flavonoids by LC-DAD-MS^2 and (c) to evaluate the antioxidant activity of lemon fruit from Tunisian origin, which has not yet been reported.

Materials and methods

Plant materials

Fruits of *C. limon* were randomly collected from healthy trees cultivated in the experimental station of the Institut National de Recherche en Génie Rural, Eaux et Forêts, Oued Souhil, Nabeul, Tunisia (latitude 36°27'53"N; longitude10°42'24"E). The fruits were picked up at three different stages of maturity based on their color, fruit size and weight in (Fig. 1). The fruits were cut on six equal portions and the flesh was removed. The flavedo and albedo layers

* Corresponding author

were carefully separated, dried at 40 °C in a forced air oven type Venticell 55 (MMM Medcenter, Einrichtungen Gmbh, Gräfelfing, Germany) to constant weight, crushed with a laboratory grinder type Blender LB20E (Waring laboratory, Torrington, USA) and subsequently essayed for their polyphenols analysis.

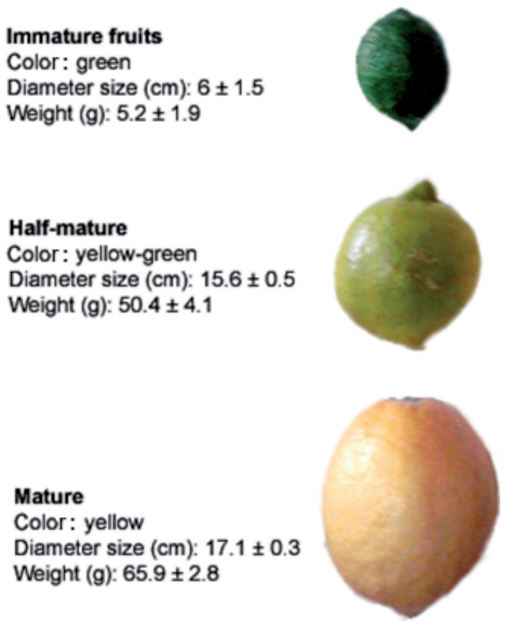

Fig. 1: Color, diameter and weight of lemon fruits at different maturity stages

Chemicals and reagents

Folin-Ciocalteu Reagent (FCR), gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox and $FeCl_3$ were purchased from Sigma-Aldrich Inc. (Steinheim, Germany). Solvents of analytical and HPLC grade were purchased from Carlo Erba Reactif-CDS (Val de Reuil, France). Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA).

Samples preparation

The extraction procedure adopted for this work is based on a simple maceration in methanol. This solvent has been largely used for the investigation of phenolic compounds in *Citrus* fruits, as it provides better extraction yields and higher efficiency compared to other solvents (Ma et al., 2008; Mokbel and Suganuma, 2006). Moreover, this procedure is sufficient enough to make comparative study on antioxidant activities of different samples. Indeed, the obtained methanolic extracts can be handled without further fractionation to perform *in vitro* assays and generate good quality analytical data, especially when using chromatographic techniques hyphenated with mass spectrometry (LC-MS or LC-MS^2).

In the present study, samples of ground powder (0.25 g) in triplicate were extracted with 100 mL of methanol (Labscan, Dublin, Ireland) for 8 h under orbital agitation (GFL 3005; Dominique Dutscher, Brumath, France). The extracts were filtered through PTFE membrane, 0.45 µm pore size (Supelco, Bellfonte, PA) and the extraction was repeated 3 times. The combined filtrates were stored at 4 °C until used.

Total phenolic content (TPC)

Total phenolics were determined with the Folin-Ciocalteu assay according to the procedure reported by McDonald et al. (2001), with slight modifications. Briefly, 500 µL of appropriately diluted extract were added to 5 mL of freshly diluted 10-fold Folin-Ciocalteu reagent, and the mixture was neutralized with 4 mL of a sodium carbonate solution (1M). The reaction mixture was kept in the dark for 15 min, and its absorbance was measured at 765 nm against a prepared methanol blank using a Jasco V-630 UV-vis spectrophotometer (Tokyo, Japan). Gallic acid was used as the standard, and results were expressed as milligram of gallic acid equivalents (mg GAE /g extract).

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The radical scavenging activity of the extracts against DPPH free radical was measured by using an aliquot of 0.2 mL of each extract that was mixed with 0.8 mL of methanol and 2 mL of a daily prepared methanol DPPH solution (0.1 mM). The mixture was shaken gently and left to stand at room temperature in the dark for 1 h. Thereafter, the absorbance was read at 515 nm. DPPH antiradical scavenging activity was extrapolated from a standard curve and expressed as micromole of Trolox per gram of extract (µM TE/g extract).

ABTS (azinobis (ethylbenzothialzoline 6-sulphonic acid)) assay

The total antioxidant activity of the samples was measured by ABTS radicals according to the method of Re et al. (1999), slightly modified. Briefly, the radical cation $ABTS^+$ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 h at room temperature. The blue-green ABTS solution was diluted in ethanol to give an absorbance of 0.7 at 734 nm prior to assay. The diluted ABTS solution (2850 µL) was mixed with 150 µL of sample extracts or trolox standard. The mixture was left to stand at room temperature in the dark for 15 min, and then the absorbance was measured at 734 nm. Trolox was used as positive control, and results were expressed as micromole of Trolox per gram of extract (µM TE/g extract).

Ferric reducing antioxidant power (FRAP) assay

The reducing power is determined by the method described by Benzie and Strain (1996), with slight modifications. Stock solutions of 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-S-triazine in 40 mM HCl), and 20 mM $FeCl_3$. $6H_2O$ were prepared. The working FRAP reagent was prepared by mixing the stock solutions in a 10:1:1 ratio. The solution was maintained at 37 °C and pH 3.6. Then, 150 µl of the sample was mixed with 2850 µl of the working solution and left standing at room temperature for 30 min in the dark. Absorbance of the mixture was then measured spectrophotometrically at 593 nm. The change in absorbance was calculated and related to the standard curve generated with trolox. Results were expressed as milligram of trolox equivalents per gram of extract (mg TE/g extract).

LC-DAD-MS^2 analysis

The chromatographic separation and mass spectrometric analyses of phenolics contained in the methanolic extracts were carried out on an Agilent 1100 series HPLC systems equipped with a diode-array detector (DAD) and a triple quadrupole mass spectrometer type Micromass Autospec Ultima Pt interfaced with an ESI ion source. Separation was achieved using a Superspher®100 (12.5 cm × 2 mm i.d, 4 µm, Agilent Technologies, Rising Sun, MD) at 40 °C. The samples (20 µL) were eluted through the column with a gradient mobile phase consisting of A (0.1 % acetic acid) and B (acetonitrile) with a flow rate of 0.3 mL/min. The following multi-step linear solvent gradient was employed: 0-5 min, 2% B, 5-60 min, 40% B,

60.1-80 min, 100% B, 80.1-110 min, 2% B, and 85.1-100 min, 2% B.

DAD detection was performed in the 250-550 nm wavelength range and the mass spectra were recorded in negative ion mode, under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 60 V; probe temperature, 350 °C; ion source temperature, 120 °C. The spectra were acquired in the m/z range of 130-750 amu.

Identification of phenolic compounds was based on detection in Multiple Reaction Monitoring (MRM) mode. All the retention times and spectral data (UV and MS spectra and MRM transitions) were compared with authentic standards and data reported from literature (Anagnostopoulou et al., 2005; Barreca et al., 2011; Nogata et al., 2006) to confirm identification.

Statistical Analysis

All measurements were carried out in triplicate and the results were presented as mean values ± SD. Statistical analyses were performed using a one-way analysis of variance ANOVA test and the significance of the difference between means was determined by Duncan's multiple range test. Correlations among data obtained were calculated using Pearson's correlation coefficient. The SPSS 18 (Chicago, Illinois, USA) and Microsoft excel package were used to perform statistical analysis.

Results and discussion

Total phenol contents

Fruit development is a dynamic process commencing with pollination and fertilization, followed by intensive cell division and expansion, with concurrent seed development and maturation. Fruit maturation typically involves tissue softening and changes in flavor, texture and color, usually caused by a series of concerted biochemical and physiological processes (Birtic et al., 2009; Hosni et al., 2011).

During the maturation of lemon fruit, the most noticeable change is the gradual decrease of total phenolic content in both flavedo and albedo peel (Fig. 2). The total phenolic contents appear to follow predictable patterns over fruit maturation, occurring at the highest levels at the immature stage (189.77 and 176.31 mg GAE/g extract in flavedo and albedo, respectively), and declining until full maturation (51.71 and 61.08 mg GAE/g extract in flavedo and albedo, respectively). Such dynamic of total phenol accumulation in flavedo and albedo tissues is consistent with that observed in *C. myrtifolia* (Barreca et al., 2011), suggesting a spatio-temporal regulation of these valuable compounds.

The decline in total phenolic contents with advancing fruit maturity has previously been reported in *Citrus limon* (Del Rio et al., 2004), *Punica granatum* (Fawole and Opara, 2013), *Malpighia emarginata* (Lima et al., 2005) and *Lycopersicon esculentum* (Ilahy et al., 2011). All these studies attribute the decrease in total phenol contents during the maturation process to the oxidation of polyphenols by polyphenoloxidase.

Usually, the observed trend was concomitant to enhanced chlorophyll degradation and a carotenoid accumulation resulting in a significant increase of yellow color. Because Lemon is a non-climacteric fruit whose ripening is regulated by abscissic acid (ABA), it may be suggested that the transition from immature (green) to mature (yellow) is mediated by this phytohormone. Support to this assumption is recently given by Palma et al. (2011) and Ren et al. (2010), who reported accumulation of ABA during the maturation of *Citrus* fruit.

This behavior is similar to that observed in some *Citrus* fruits such as Satsuma mandarin, Valencia orange and Lisbon lemon (Kato et al., 2004). From the molecular point of view, the massive

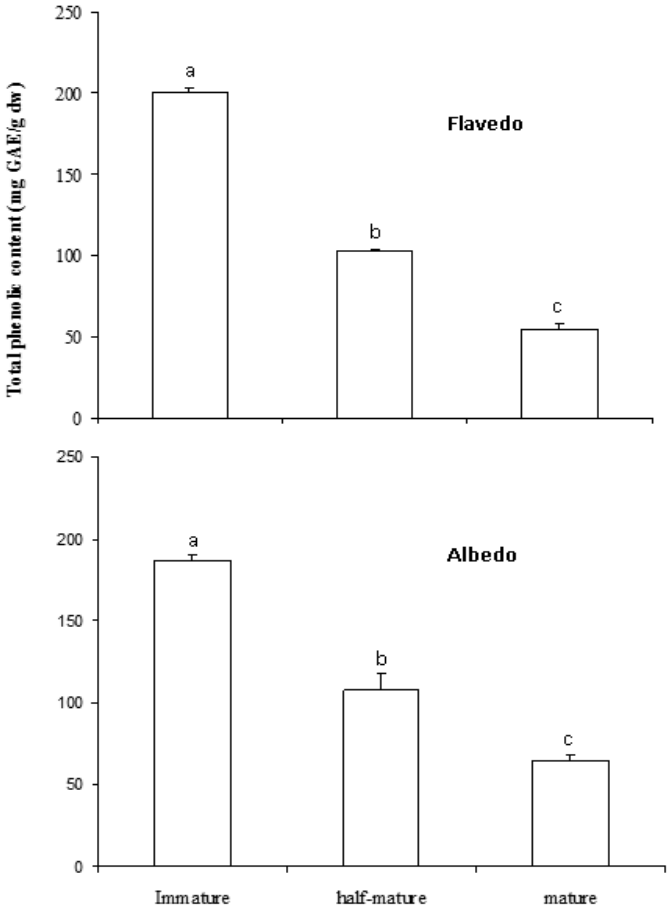

Fig. 2: Total phenolic contents (mg GAE /g extract) at different stages of maturity. Different letter(s) on bar indicate statistically significant differences ($p<0.05$).

accumulation of carotenoids (mainly lycopene) was mainly due to a significant up-regulation of the gene expressions namely phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) during fruit maturation, resulting in enhanced lycopene synthesis (Schweiggert et al., 2011).

From quantitative standpoint, our obtained values were different from those reported in some *Citrus* species. For example, the albedo layer of Argentinean *C. aurantium* was found to contain variable amounts (234-322 mg GAE/g extract) of total phenolic contents, depending on the extraction procedure (Zapata Zapata et al., 2012). Similarly, Ghanem et al. (2012) have compared the total phenolic content in three Tunisian *Citrus* species, and have reported values varying from 29.11 and 24.51 mg cafeic acid equivalent/g dw in *C. reticulata* and *C. limon*, respectively, to 18.99 mg cafeic acid equivalent/g dw in *C. sinensis*. In another comprehensive study from Mauritius, Ramful et al. (2011) compared the total phenol content in 20 varieties belonging to 11 *Citrus* species and reported values ranging from 406.3 to 1694 µg GAE/g fresh weight. In general, the observed discrepancy between results may be due to the genetic makeup, origin, peel part analyzed, stage of maturity, environmental conditions, samples preparation and analysis. On the other hand, the high concentration of phenolic compounds in *Citrus* peel was primarily ascribed to their protective role against UV radiations, pathogens and predators (Barros et al., 2012).

Polyphenols are known as potent antioxidant; thereby any changes in their contents may have strong influence on their biological activities. In this direction, the flavedo and albedo extracts at different stages of fruit maturity were evaluated for their antioxidant activities.

Antioxidant activity

Scavenging activity on DPPH radicals

The DPPH radical is a stable radical with absorption band at 515-528 nm. It loses this absorption when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow. Because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening antiradical activities of extracts (DZIRI et al., 2012).

Fig. 3 shows that irrespective to the peel parts, the scavenging activity on DPPH radicals of all extracts was the highest (55.91 and 56.76 µM TE/g extract for flavedo and albedo, respectively) at the immature stage, and then decreased gradually to reach the lowest values (23.07 and 26,04 µM TE/g extract for flavedo and albedo, respectively) at the full maturation stage. The hydrogen-donating ability of flavedo and albedo extracts is most likely attributed to their higher total phenolic contents. A strongly significant (p<0.01) positive correlation (r = 0.986) was detected between total phenolic contents and the DPPH radical scavenging activity, supporting thereby our hypothesis and confirm previous reports that free radical scavenging activity can be related to phenolic content (DUDONNE et al., 2009).

Scavenging activity on ABTS radicals

For ABTS radicals scavenging activity (Fig. 4), the rank order among the extracts was similar to DPPH results. The immature flavedo and albedo peel extracts showed the highest antioxidant activities (87.21 and 82.01 µM TE/g extract for flavedo and albedo, respectively), followed by the extracts from the half-mature fruits (54.02 and 57.77 µM TE/g extract for flavedo and albedo, respectively), while the extracts from the full mature fruits exhibited the lowest antioxidant capacities (32.28 and 50.52 µM TE/g extract for flavedo and albedo, respectively).

As for the DPPH assay, significant (p<0.01) linear correlation (r = 0.851), was found between ABTS assay and total phenolic content, confirming the relationships between phenolic compounds concentration in plant extracts and their radical scavenging activity. Similar to the results reported herein, XU et al., 2008 evaluated the ABTS radical scavenging capacity of 15 varieties of Chinese citrus and found that the antioxidant activity was associated with their higher total phenolic contents.

Another point to be considered is that ABTS values are significantly higher than the DPPH values. The lower values determined by the DPPH assay may be due to color interference by carotenoids (λ_{max}= 400-500 nm) and anthocyanins (λ_{max}= 470-580 nm) with DPPH

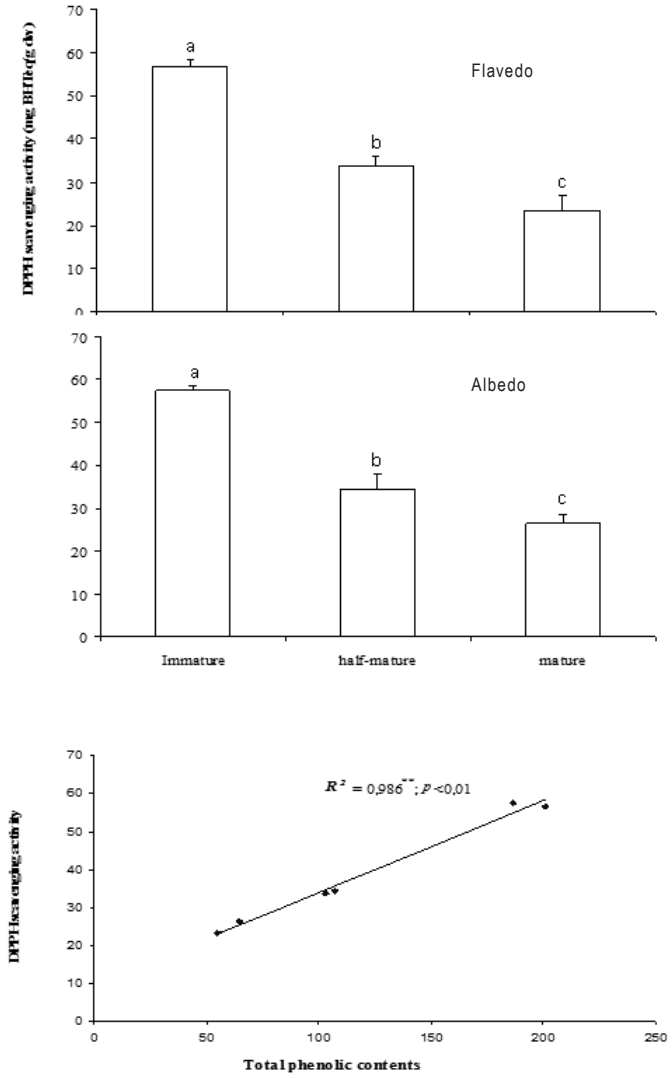

Fig. 3: DPPH radicals scavenging activity of lemon flavedo and albedo extracts (in µM TE/g extract) and its correlation with total phenolic contents. Different letter(s) on bar indicate statistically significant differences (p<0.05); ** significance at p<0.01.

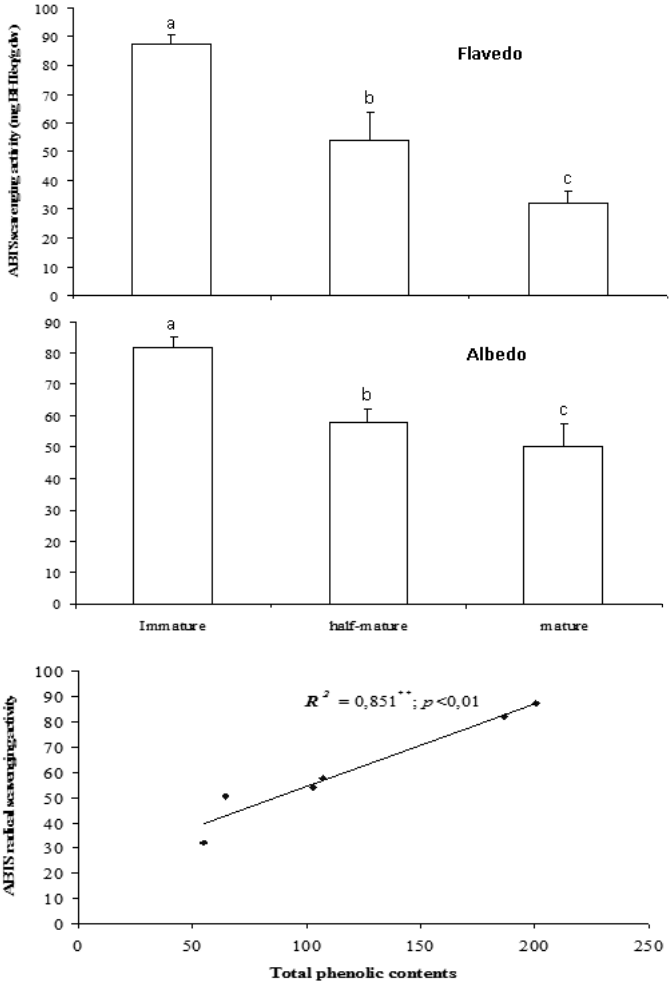

Fig. 4: ABTS radicals scavenging activity of lemon flavedo and albedo extracts (in µM TE/g extract) and its correlation with total phenolic contents. Different letter(s) on bar indicate statistically significant differences (p<0.05); ** significance at p<0.01.

chromagen (λ_{max}= 515 nm). These results were in good agreement with previous works on other species such as guava (THAIPONG et al., 2006) and potato (TEOW et al., 2007).

Ferric reducing antioxidant power (FRAP)

Results of FRAP assay are shown in Fig. 5. The trend for the ferric ion reducing activities of the flavedo and albedo peel extracts did not vary markedly from their DPPH and ABTS scavenging activities. In this study, extracts from the immature fruits possessed the highest ferric reducing capacity (241.71 and 297.11 mg TE/g extract for flavedo and albedo, respectively), while the lowest capacity was exhibited by extracts from full mature fruits (58.06 and 70.44 mg TE/g extract for flavedo and albedo, respectively). These results were in good agreement with those of BARRECA et al. (2011) who reported the strong antioxidant activity of *C. myrtifolia* peel extracts, with the albedo extracts being the most powerful when compared with flavedo ones.

Alike the DPPH and ABTS assays, the ferric reducing capacity was significantly (p<0.01) correlated with the total phenolic contents (r = 0.918), which indicates that the reducing power of flavedo and albedo extracts were mainly attributable to their phenolic compounds. These studies were in line with those reported for some *Citrus* species such as *C. sinensis, C. reticulata, C. latifolia, and C. limettioides* (BARROS et al., 2012), where the ferric reducing power was found to be tightly associated with higher total phenolic contents. In contrast, a very weak ferric reducing power was found in *C. reticulatae pericarpium* and *C. reticulatae viride pericarpium* from Taiwan (SU et al., 2008). Such divergences can be due to different cultivars/varieties, origin, extraction methods and the analytical procedures (RAMFUL et al., 2011).

In any case, total phenolic contents in lemon peels can be used as indicator in assessing the antioxidant activity since they showed high correlations with all assays (DPPH, ABTS, and FRAP). Also, high correlations between DPPH and ABTS (r = 0.938, p<0.01), DPPH and FRAP (r = 0.973, p<0.01), and ABTS versus FRAP (r = 0.879, p<0.01) were also found (Fig. 6). These results indicate that flavedo and albedo extracts had comparable activities in all the three assays, and are consistent with the view that the three assays share a similar mechanistic basis, especially transfer of electrons from the antioxidant to reduce an oxidant, as proposed by CLARKE et al. (2013).

Additionally, the integral lemon peel (flavedo + albedo) may be considered as valuable source of antioxidant phenolic compounds, because of their ability to inhibit free radicals formation and to suppress the formation of the Fenton reaction, and hence, impede the formation of a highly reactive hydroxyl radical (PICHAIYONGVONGDEE and HARUENKIT, 2009).

Bearing in mind that the antioxidant activity of plant extracts is directly related to their chemical composition and the structural conformation of their phenolic compounds (JANG et al., 2010), it is of interest to characterize the phenolic constituents of both flavedo and albedo extracts.

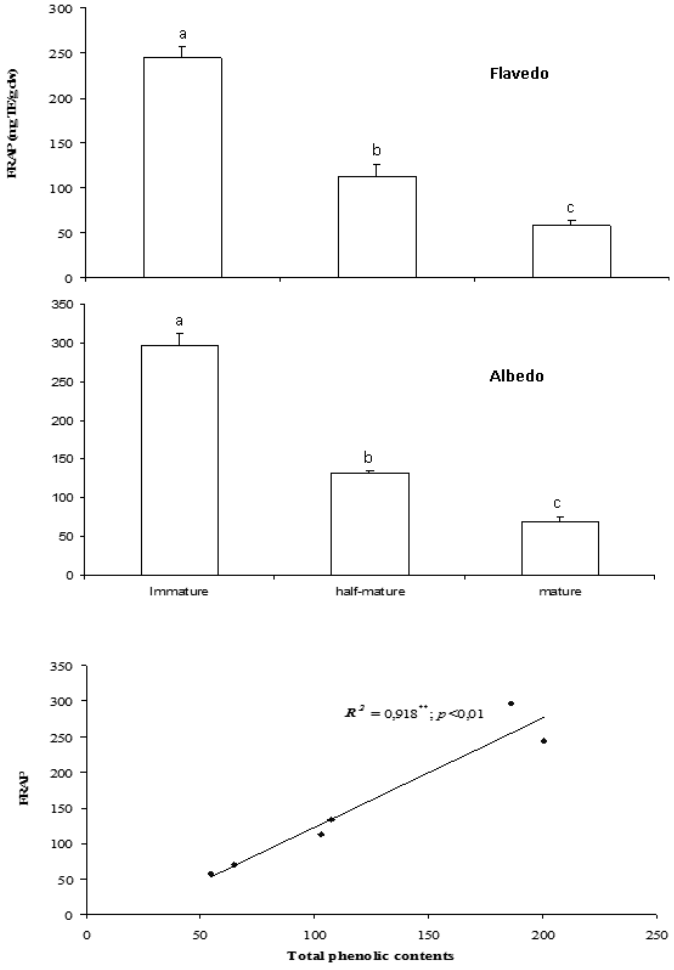

Fig. 5: Ferric reducing antioxidant power (FRAP) of lemon flavedo and albedo extracts (in mg TE/g extract) and its correlation with total phenolic contents. Different letter(s) on bar indicate statistically significant differences (p<0.05); ** significance at p<0.01.

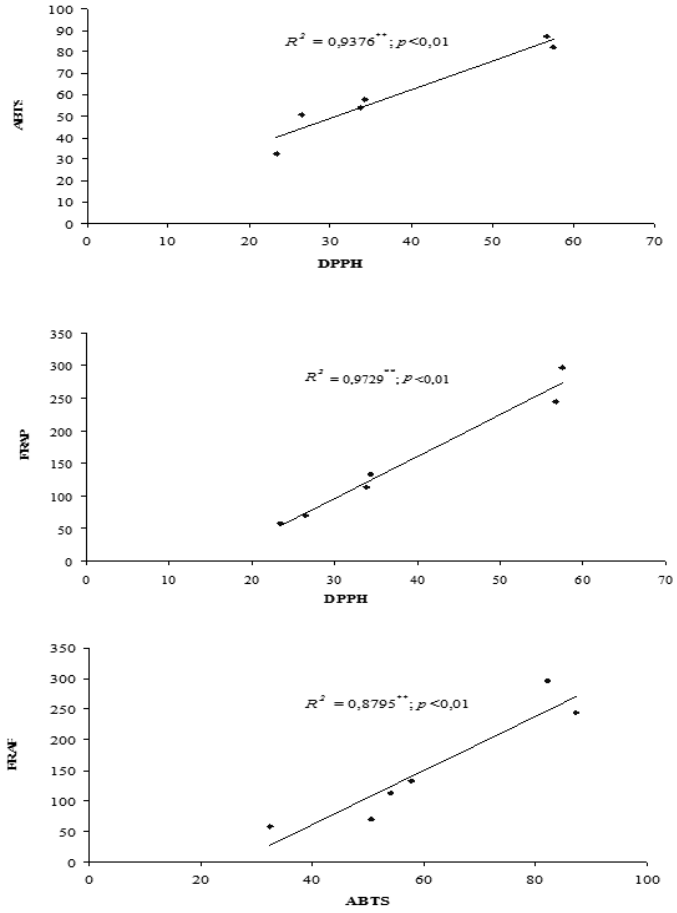

Fig. 6: Coefficients of determination between DPPH, ABTS and FRAP; ** significance at p<0.01.

Identification of phenolic components by LC-DAD-MS/MS

The typical phenolic profile of lemon peel (flavedo part) is depicted in Fig. 7a. Data of the retention time (*tr*), UV, mass spectra and MRM transitions of the identified compounds are given in Tab. 1.

As can be seen, 5 components (peaks 4-8) were unambiguously identified by comparing their retention time *tr*, UV, MS spectra and MRM transitions with those of reference standards (Fig. 7b). Peak 4 (*tr* = 28.58 min, λ_{max} : 286 nm) exhibited $[M-H]^-$ base ion at m/z 595 and fragmentation ion at m/z 151. By comparing its tr, UV spectrum, molecular mass and fragmentation pattern with the reference standard, this compound was identified as eriocitrin (Eriodictyol-7-*O*-rutinoside) (BARRECA et al., 2011). Peak 5 (*tr* = 31.01 min; λ_{max} : 256-354 nm) showed a $[M-H]^-$ ion at m/z 609 with a base peak at m/z 301 indicative of the quercetin aglycone. This compound was assigned as rutin (quercetin-3-*O*-rutinoside) by comparison of retention time, UV spectrum and mass spectrometric data with rutin reference standard (HOSNI et al., 2013). Peak 6 (*tr* = 31.55 min; λ_{max}: 256-354 nm) showed a pseudo-molecular ion $[M-H]^-$ at m/z 463 and a fragment [M-H-162] ion at m/z 301 (quercetin aglycone) due to the elimination of hexose unit (162 amu). This compound was identified as quercetin-3-*O*-galactoside (hyperoside) by comparison of the retention time, UV, mass spectra and specific transitions with authentic standard (ALBOUCHI et al., 2013; DE BRITO et al., 2007). Peak 7 (*tr* = 35.11 min; λ_{max}: 286 nm) exhibited $[M-H]^-$ base ion at m/z 609 and a fragment [M-H-308] ion at m/z 301. Coelution with a known standard, along with comparison with literature data (BARRECA et al., 2011), led to the identification of peak 7 as hesperidin, frequently reported as the main components of lemon. Peak 8 (*tr* = 36.18 min; λ_{max}: 253-346 nm) exhibited $[M-H]^-$ base ion at m/z 607 and a fragment [M-H-308] ion at m/z 299. This fragmentation pattern is similar to that observed for the diosmin reference standard and literature data (NOGATA et al., 2006) .

Unfortunately, for the remaining components; peak 1 (*tr* = 23.8 min; λ_{max}: 349 nm, $[M-H]^-$ 609), peak 2 (*tr* = 25.49 min; λ_{max}: 271-349 nm; $[M-H]^-$ 593), peak 3 (*tr* = 27.4 min; λ_{max}: 271-247 nm, $[M-H]^-$ 479) and peak 9 (*tr* = 36.9 min; λ_{max}: 361 nm, $[M-H]^-$ 56=651), the UV spectrum and mass spectra are not sufficient to elucidate their structure and they are still unidentified.

Previous studies have shown that hesperidin and eriocitrin, a flavanone glucosides, were the major flavonoids in the pulp of lemon (*C. limon*) and lime (*C. aurantiifolia*) (PETERSON et al., 2006). The former compounds have also been reported as the most prominent flavanone in *C. sinensis* and the interspecific hybrid *C. reticulata* × *C. sinensis* (GOULAS and MANGANARIS, 2012) and *C. latifolia* (DA SILVA et al., 2013). The flavone glycoside, diosmin and the flavonol rutin have been frequently reported in *C. limon* (BALDI et al., 1995), *C. sinensis* (ANAGNOSTOPOULOU et al., 2005), *C. reticulata, C. tankan,* and *C. grandis* (WANG et al., 2008). In contrast, the occurrence of hyperoside in lemon was reported herein for the first time.

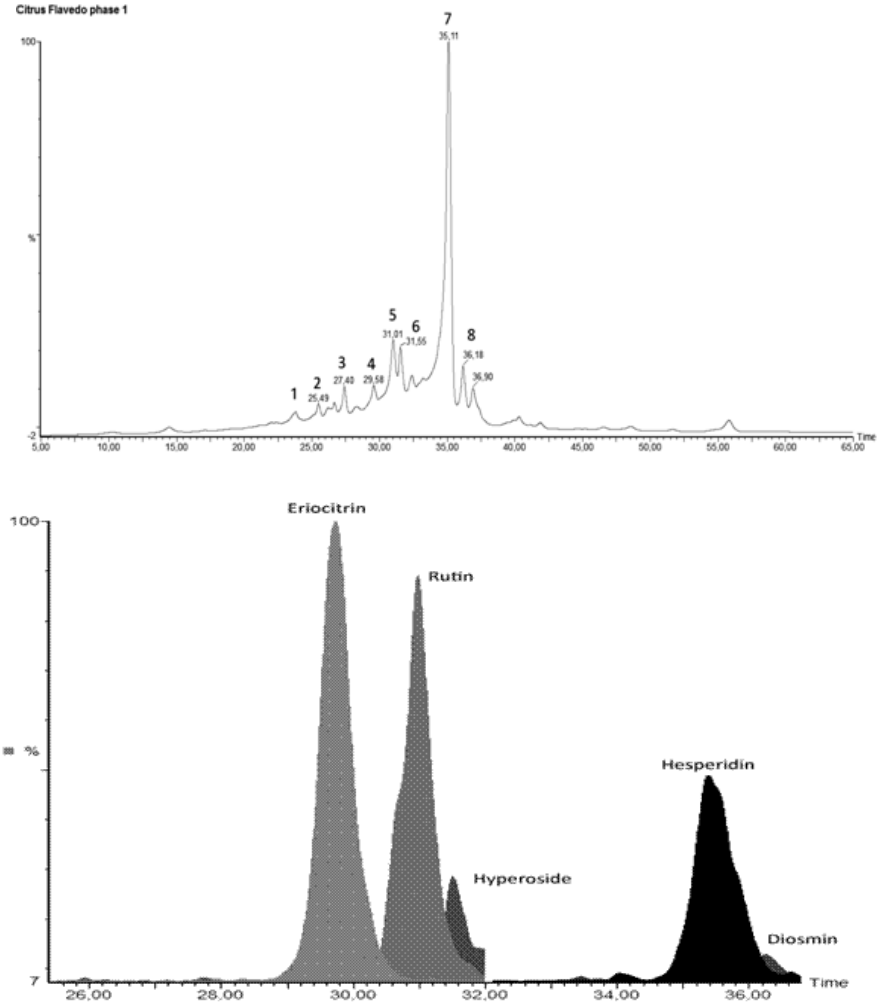

Fig. 7: Typical LC-MS/MS chromatogram of lemon peel methanolic extracts (a) and MRM chromatogram of the identified components (b) (peak assignments are given in Tab. 1.

Tab. 1: HPLC retention times, UV spectra, ESI/MS (-) (m/z), MS/MS transition and tentative identification of phenolic components from lemon peel methanolic extract

Peak N°	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$	MS/MS transitions	Identified compounds
4	29.58	286	595	595.5 > 151.2	Eriocitrin
5	31.01	256; 354	609	609.4 > 300.2	Rutin
6	31.55	256; 354	463	463.2 > 300.2	Hyperoside
7	35.11	286	609	609.57 > 301.2	Hesperidin
8	36.18	253; 346	607	607.5 > 299.2	Diosmin

Conclusion

The antioxidant activity of the identified components has previously been reported. For example, hesperidin was found as the main contributor to the antioxidant activity of peel and juice of different *Citrus* species (Di Majo et al., 2005). According to the latter authors, the potent antioxidant and free radical scavenging activity of hesperidin may be ascribed to the hydroxyl group at the position 3'. The scavenging activity of hesperidin on DPPH radicals, hydroxyl radicals, hydrogen peroxide, superoxide anions and its reducing power has been evidenced three years later by Yi et al. (2008). For eriocitrin, the first evidence for its antioxidant activity has been reported by Miyake et al. (1997), who successfully isolated this component from lemon fruits and proved its antioxidant activity by using the linoleic acid system. Six years later, the same group of researchers have reported the protective effects of eriocitrin against oxidative damages caused by acute exercise-induced oxidative stress (Minato et al., 2003), In another study from Poland, Sroka et al. (2005) have reported that eriocitrin possess strong scavenging activity against DPPH radicals and hydrogen peroxides. In contrast, they found a very weak antioxidant activity of the flavone diosmin, supporting the previous results by Castillo et al. (2000). The latter authors attributed the weaker antioxidant activity of diosmin to the methylation of the 4'-hydroxyl group in the B-ring, which significantly reduces the antioxidant of this component, *via* the reduction of its electron-donating ability.

The glucolsylated flavonols, rutin and hyperoside have been found to possess strong antioxidant activity because of its adjacent dihydroxy groups on the B-ring (Anagnostopoulou et al., 2005; Rainha et al., 2013; Yang et al., 2008).

Taken together, it appears that the strong antioxidant activity of lemon peel can be attributed to specific phenolic components acting alone or in combination. Taking into consideration the complexity of the antioxidant mechanism, which includes ***i***) single electron transfer from the antioxidant to the radical leading to indirect H-abstraction (Rojano et al., 2008), ***ii***) direct hydrogen atom transfer from the antioxidant to the radical (Mayer and Rhile, 2004), ***iii***) sequential proton-loss electron transfer (Klein and Lukeš, 2007) and ***iv***) metal chelating (Gülçin et al., 2010), it is difficult to get deeper insight into the antioxidant mechanism of lemon peels and to ascertain the exact contribution of their individual phenolic compounds. Nevertheless, the results of this study could be helpful to find possible nutritional, cosmeceutical and pharmaceutical outlets for the discarded fruit parts.

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Address of the corresponding author:
E-mail: nadya.mcharek@gmail.com