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CHARACTERIZATION OF PHENOLICS CONTENT AND ANTIOXIDANT ACTIVITY OF LEAVES FROM ORANGES CULTIVATED IN ALGERIA

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Abstract

Description of the subject: Orange leaves have always had an important place in the therapeutic arsenal of humanity. Few studies have been carried out on the leaves of certain blond and pigmented varieties. In Algeria no study has been published; our interest in these products comes from these observations.
Object: The objective of this research is to determine and compare the antioxidant activity of leaves of seven varieties of orange grown in Algeria and characterize the composition of phenolic compounds by HPLC-DAD analysis.
Methodology: Free phenolic acid and flavonoid profiles of the orange cultivars were analyzed by HPLC-DAD, whereas antioxidant capacities were evaluated in vitro using scavenging assay of hydrogen peroxide (HPS), phosphomolybdate method (PMM) and ferrous ion chelating ability (FIC).
Results: Amid the tested cultivars, Bigarade possessed the strongest antioxidant capacities. Two phenolic acids and seven flavonoids were identified and quantified. Kaempferol was the major flavonoids in *C. sinensis* L. However, apigenin-7-glycoside and rutin constituted the greater part of total flavonoids in *C. aurantium* L, respectively.
Conclusion: This information will probably be useful for the utilization of orange leaves as antioxidants in food and drug preparation.
Key words: Antioxidant capacity, characterization, leaves, orange, phenolic profile.

CARACTÉRISATION DU CONTENU PHÉNOLIQUE ET DE L'ACTIVITÉ ANTIOXYDANTE DES FEUILLES D'ORANGERS CULTIVÉS EN ALGÉRIE

Résumé

Description du sujet: Les feuilles d'oranger ont toujours eu une place importante dans l'arsenal thérapeutique de l'humanité. Peu d'études ont été menées sur les feuilles de certaines variétés blondes et pigmentées. En Algérie, aucune étude n'a été publiée notre intérêt pour ces produits découle de ces constatations.
Objectif: L'objectif de cette recherche est de déterminer et de comparer l'activité antioxydante des feuilles de sept variétés d'oranges cultivées en Algérie et de caractériser la composition phénoliques par analyse HPLC-DAD.
Méthodologie: Les profils des acides phénoliques libres et de flavonoïdes ont été analysés par HPLC-DAD, tandis que les capacités antioxydantes ont été évaluées *in vitro* en utilisant un test de piégeage du peroxyde d'hydrogène (HPS), la méthode au phosphomolybdate (PMM) et la capacité de chélation des ions ferreux (FIC).
Résultats: Deux acides phénoliques et sept flavonoïdes ont été identifiés et quantifiés. Le kaempférol était le principal flavonoïde de *C. sinensis* L. Cependant, l'apigénine-7-glycoside et la rutine constituaient la plus grande partie des flavonoïdes totaux de *C. aurantium* L, respectivement. Parmi les cultivars testés, Bigarade possédait les capacités antioxydantes les plus fortes.
Conclusion: Ces informations seront probablement utiles pour l'utilisation des feuilles d'oranger comme antioxydants dans la préparation des aliments et des médicaments.
Mots clés: capacité antioxydante, caractérisation, feuilles, orange, profil phénolique.

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INTRODUCTION

Citrus fruits are the most cultivated fruits in the world. Orange is the most important species of citrus. The importance of this fruit is attributed to its good taste quality, its richness in nutrients essential for the organism and its diversified uses (raw fruit, in the form of jam or juice) [1]. The beneficial effects of this fruit are mainly due to the presence of bioactive compounds such as phenolic compounds, vitamin C, carotenoids and essential oils [2].

Orange leaves have always had an important place in the therapeutic arsenal of humanity. According to Bruneton [3], they have been used traditionally in the symptomatic treatment of neurotonic conditions in adults and children, especially in cases of minor sleep disorders. They are also used for the extraction of essential oils which are used to protect the human body from damage caused by free radicals and to delay the progression of several chronic diseases [4].

Few studies have been carried out so far on the by-products of orange trees, especially on the leaves and peels of certain blond and pigmented varieties. The themes dealt with concern in particular the analysis of the chemical composition, the identification of the lipid fraction and the study of the antimicrobial and antioxidant activities of certain varieties of oranges (Navels and bitters) [5, 6, 3, 7]. Unfortunately, in Algeria no study has been undertaken. Our interest in these products comes from these observations and the present work consists in filling the lack of information on the properties of the leaves of orange trees of local origin.

The objective of this research is to determine and compare the antioxidant activity of leaves of seven varieties of orange (Washington Navel, Thomson Navel, Sanguinelli, Double Fine, Portuguese, Jaffa and Bitter) grown in Algeria and characterize the composition of phenolic compounds by HPLC-DAD analysis.

MATÉRIEL ET MÉTHODES

1. Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all work. HPLC-grade acetonitrile, formic and sulfuric acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45 µm pore size membrane filter. Ferulic and gallic acids, narirutin, naringin, hesperidin, poncirin, rutin, kaempferol and apigenin-7-glycoside were purchased from Sigma–Aldrich (Steinheim, Germany). All other reagents and chemicals were purchased from Biochem Chemopharma (United kingdom).

2. Preparation of sample

The leaves of seven varieties of oranges were harvested from the region of Bejaia (Timezrit and Amizour) (North East of Algeria). The cultivars were: sweet orange (Thomson, Washington, Sanguinelli, Double Fine, Portugaise and Jaffa) and sour orange (Bigarade**)**.

3. Extraction and HPLC analysis of phenolic compounds

3.1. Extraction and purification of phenolic compounds

3g of dried powder were extracted with 30 ml of methanol-water (80%) at room temperature for 22 hours using magnetic blender. Then, the extract was vacuum filtered through sintered glass filter crucibles (porosity 3) and the residue was taken up again with 30 ml of acetone-water (70%). The volumes of the two obtained filtrates were mixed and then centrifuged at 3060 g for 5 minutes (Sigma 2-16K, Osterode, Germany) and vacuum filtered using Whatman N°. 1 paper. The obtained aqueous organic extract was concentrated, under reduced pressure at 40°C using a rotary evaporator (Rotavapor R-200/205, Buchi, Flawil, Switzerland).

The aqueous extract was washed with oil ether to eliminate the pigments (chlorophylls and carotenoids) then washed with the ethyl acetate. After a strong agitation and a decantation, the phenolic compounds pass in the ethyl acetate and sugars remain in the aqueous phase. Each operation was repeated 4 times. The organic phase was recovered and concentrated in rotary evaporator at 40°C, until complete evaporation and then reconstituted in pure methanol.

3.2. Analysis of phenolic compounds

Analysis were performed in triplicate on a Agilent 1100 HPLC system (Agilent Technologies, Palo Alto CA-USA) operated by Windows NT based ChemStation software equipped with a diode array detector (DAD), binary pump, degasser and auto sampler. The column used was a Beckman Ultrasphere ODS (Roissy CDG, France): 4.6 mm×250 mm, 5 µm equipped with a precolumn 4.6 mm×10 mm (same granulometry). The mobile phase consisted of two solvents: Solvent A, water/formic acid (95/5; v/v) and Solvent B, acetonitrile/solvent A (60/40; v/v).

Phenolic compounds were eluted under the following conditions: 1ml/min follow rate and the temperature was set at 25°C, isocratic conditions from 0 to 10 min with 0% B, gradient conditions from 0 % to 5 % B in 30 min, from 5 % to 15 % B in 18 min, from 15 % to 25 % B in 14 min, from 25 % to 50 % B in 31min, from 50% to 100% B in 3 min, followed by washing and reconditioning the column. The ultraviolet–vis spectra (scanning from200 to 600 nm) were recorded for all peaks. The identification of phenolic compounds were obtained by comparing the retention times and ultra-violet–visible spectra with authentic standards and with previously reported data in the literature [8]. The quantification of each identified compounds was performed on each samples using an external standard calibration curve for each compound. The curves were obtained using the commercial standards of the concentrations normally present in extracts (approximately 1–100 mg kg^{-1}), obtaining regression coefficients (R2) above 0.995 in all cases.

4. Antioxidant activity

4.1. Phosphomolybdenum method (PMM)

The total antioxidant capacities of the sample extracts were evaluated by the phosphomolybdenum method as described by Prieto et *al.* [9]. Quercetin and gallic acid were used for comparison at the concentration 0.2 mg/ml.

4.2. Hydrogen peroxide scavenging capacity (HPS)

Hydrogen peroxide scavenging ability of the peels and leaves extracts was performed according to the method of Kumar et *al.* [10]. Quercetin and gallic acid were used for comparison at the concentration 0.1 mg/ml.

A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

4.3. Ferrous ion chelating capacity (FIC)

The ferrous ion chelating capacity was determined as described by Bhandari and Kawabata [11]. EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50% and was obtained by interpotation from linear regression analysis. EDTA and quercetin were used for comparison at the concentration 2.4 mg/ml and 2.4µg/ml, respectively.

5. Statistical analysis

Experiments were conducted in three parallel measurements and results were expressed as mean ± standard deviation (SD). The averages and the standard deviations are calculated with Microsoft Office Excel 2010. The software STATISTICA 5.5 was used to compare the different results by the analysis of variance with one and two factors (ANOVA). Differences were considered to be significant at $p \leq 0.05$.

RÉSULTATS

1. Phenolic contents of the samples

The composition and concentrations of major phenolic substances that were determined by HPLC – DAD analysis are presented in Table 1. Two free phenolic acids were identified, including gallic acid and ferulic acid. In the group of phenolic acids, gallic acid was found in all cultivars but ferulic acid was found only in leaves of Washington, Double fine and portugaise. Gallic acid content varied significantly ($p \leq 0.05$) between cultivars, and ranged from 22.52 µg/g DM (Sanguinelli) to 535.92 µg/g DM (Double fine). On the other hand, the proportion of ferulic acid varied significantly ($p \leq 0.05$) between cultivars, and ranged from 69.94µg/g DM (Double fine) to 147.08 µg/g DM (Washington).

As shown in Table 1, the content of flavonoids identified were significantly different ($p \leq 0.05$) among the orange cultivars. Interestingly, analysis of the profiles of polyphenols by HPLC-DAD has seven quantization flavonoids that have not yet been quantified in the orange leaves and especially Algeria orange by product. These flavonoids are narirutin, naringin, hesperidin, poncirin, kaempferol, rutin and apigenin -7- glycoside (AP7G).

The obtained results show that the flavones (AP7G) and the flavonols (kaempferol and rutin) neveals a ubiquitous distribution pattern compared to that of flavanons glycosids (narirutin, naringin, hesperidin and poncirin) for peels and leaves.

2. Antioxidant activity

The analysis of the reducing power at concentration of 1 mg/ml, with the phosphomolybdenum method, resulted absorbances between 0.27 to 0.44. As can be seen in table 2, it is the Bigarade and portugaise varieties that have the highest absorbance and subsequently have the most pronounced reducing power, but significantly lower

($p \leq 0.05$) than that of the gallic acid and quercetin used as standard for quantification.

Table 1: Content of individual phenolic compounds of leaves from selected orange varieties determined by HPLC-DAD (µg/g DM)

	Cultivars						
	Washington	Thomson	Sanguinelli	Double fine	Portugaise	Jaffa	Bigarade
Gallic acid	82.68±0.62^{D}	31.34±0.24^{F}	22.52±0.16^{G}	535.92±4.02^{A}	40.94±0.30^{E}	355.9±2.68^{B}	249.26±1.88^{C}
Ferulic acid	147.08±1.1^{A}	Nd	Nd	69.94±0.52^{C}	111.72±0.84^{B}	Nd	Nd
TPA	299.76	31.34	22.52	605.86	152.66	355.9±2.68	249.26
Narirutin	55.74±0.42^{E}	Nd	88.56±0.66^{D}	124.08±0.94^{C}	88.46±0.66^{D}	251.88±1.9^{B}	377.52±2.84^{A}
Naringin	Nd	Nd	Nd	80.68±0.60^{C}	Nd	184.08±1.38^{B}	227.8±1.72^{A}
AP7G	39.06±0.3^{G}	51.68±0.38^{E}	62.32±0.46^{D}	64.00±0.48^{C}	49.58±0.38^{F}	199.52±1.5^{B}	408.74±3.06^{A}
Hesperidin	64.4±0.48^{D}	Nd	68.78±0.52^{C}	Nd	Nd	293.62±2.20^{A}	77.96±0.58^{B}
Poncirin	116.88±0.88^{C}	390.18±2.94^{A}	108.52±0.82^{D}	209.02±1.56^{B}	Nd	93.92±0.70^{E}	Nd
Rutin	185.88±1.4^{D}	492.36±3.70^{B}	142.84±1.08^{E}	139.04±1.04^{F}	76.14±0.58^{G}	361.6±2.72^{C}	639.98±4.80^{A}
Kaempferol	1466.96±11.02^{A}	781.12±5.86^{D}	984.40±7.4^{C}	622.86±4.68^{F}	666.56±05.00^{E}	1163.54±8.74^{B}	184.28±1.38^{G}
TF	1928.92	1715.34	1455.42	1239.68	880.74	2548.16	1916.28

AP7G: Apigenin7 glycosids. DM: dry matter. Nd: Not determined. TF: Total flavonoids. TPA: Total phenolic acids. Each value in the table is the mean ± standard deviation (n = 3).Values in the same row sharing different letters are significantly different (p<0.05). The results are sorted in decreasing order: A >B >C >D>E >F >G.

Table 2: Antioxidant activities of leaves from selected orange varieties and standards

Cultivars / standards	PMM (absorbance)	HPS (%)	FIC (%)
Washington	0.34±0.01^{E}	93.54±1.35 ^{B}	75.39±0.40 ^{D}
Thomson	0.39±0.01^{D}	94.87±1.13^{AB}	87.19±0.59 ^{B}
Sanguinelli	0.33±0.01^{E}	89.87±0.41 ^{C}	73.46±0.17 ^{E}
Double fine	0.31±0.00 ^{F}	86.61±0.75 ^{D}	62.25±0.40^{H}
Portugaise	0.44±0.01^{C}	95.32±0.81^{AB}	71.75±0.67^{F}
Jaffa	0.27±0.00 ^{G}	86.57±0.71 ^{D}	69.30±0.65 ^{G}
Bigarade	0.44±0.01^{C}	96.19±0.53 ^{A}	88.49±0.53^{B}
Gallic acid	0.97±0.01 ^{A}**	90.00±0.20 ^{C}*	
Quercetin	0.76±0.00 ^{B}**	67.04±0.10 ^{E}*	81.89±0.34 ^{C} ***
EDTA			97.67±0.28 ^{A} ****

PMM: phosphomolybdate method. FIC: Ferrous ion chelating capacity. HPS: Hydrogen peroxide scavenging capacity. Each value in the table is the mean ± standard deviation (n = 5).
Values for the same method sharing different letters are significantly different (p<0.05). The results are sorted in decreasing order: A >B >C >D>E >F >G >H.
** at 0.1 mg/ml. ** at 0.2 mg/ml. *** at 2.4 mg/ml.**** at 2.4µg/ml*

Concerning the ferrous ion chelating capacity of samples tested at the concentration of 12 mg/ ml, the results consigned in table 2 revealed clearly that significant differences ($p \leq 0.05$) in the FIC power were noticed between the cultivars. The FIC capacity ranged from 62.25 to 88.49 %. Thomson and Bigarade varieties exhibited the highest antioxidant capacity (87.19 and 88.49%, respectively), whereas Double fine and Jaffa cultivars show the lowest FIC capacity (62.25 and 69.30 %, respectively). According to the data, it can be seen that whatever the variety and the considered part of the plant is, the EDTA which proves the most FIC efficiency. Ferrous ion chelating activity of the quercetin is statistically inferior ($p < 0.05$) than that of leaves of Bigarade and Thomson.

The hydrogen peroxide scavenging capacity values for the investigated extracts ranged from 86.57 to 96.19 %. Bigarade presents the highest inhibition activity (96.19%). The comparison of mean total antioxidant activity of leaves samples indicate that it is the leaves that exhibit the greatest scavenging capacity. The latter is statistically superior ($p \leq 0.05$) than that of quercetin but significantly inferior ($p \leq 0.05$) than that of gallic acid for the varieties Double fine and Jaffa, and similar ($p > 0.05$) than that of Sanguinelli.

The calculated IC_{50} and EC_{50} are reported in the Table 3. Low IC_{50} corresponds to a strong inhibitory capacity of H_2O_2. The values indicate that the IC_{50} of quercetin is significantly lower ($p \leq .05$) followed by gallic acid, leaves of Bigarade, Portugaise, Thomson and Washington. The data shows that the EC_{50} of the EDTA is significantly lower ($p \leq 0.05$) followed by the quercetin, leaves of Bigarade and Thomson. The highest EC_{50} is recorded for the leaves of red varieties and Jaffa.

Table 3: IC_{50} of the H_2O_2 Scavenging capacity and EC_{50} of Chelating effect of leaves and standards

	IC_{50} (mg/ml)	**EC_{50}** (mg/ml)
	HPS (%)	FIC (%)
Washington	0.552±0.031 ^{CD}	7.837±0.444 ^{D}
Thomson	0.550±0.028 ^{CD}	6.819±0.450 ^{C}
Sanguinelli	0.582±0.030 ^{DE}	8.295±0.555 ^{DE}
Double fine	0.620±0.048 ^{EF}	9.484±0.350 ^{FG}
Portugaise	0.542±0.050 ^{CD}	8.480±0.500 ^{DE}
Jaffa	0.628±0.032 ^{EF}	8.717±0.657 ^{DEF}
Bigarade	0.525±0.022 ^{C}	6.785±0.445 ^{C}
Gallic acid	0.074±0.005 ^{B}	
Quercetin	0.056±0.003 ^{A}	1.475±0.015 ^{B}
EDTA		0.0013±0.0003 ^{A}

IC50: Concentration of sample required for 50% inhibition.EC 50: Concentration at which ferrous ions were chelated by 50%.FIC: Ferrous ion chelating capacity. HPS: Hydrogen peroxide scavenging capacity. Each value in the table is the mean ± standard deviation (n = 3).
Values for the same method sharing different letters are significantly different ($p<0.05$). The results are sorted in crescent order: A <B <C <D<E <F <G.

3. Correlation

The results presented in this study (Table 4) showed a very weak correlation between the flavonoid contents of leaves and, the FIC, HPS and the PMM assays (0.057, 0.052 and 0,188 respectively). Implying that the flavonoids may not be the main components responsible for HPS ability and FIC of the tested extracts. On the other hand, a weak correlation is observed also between the phenolic acids contents of leaves and the FIC, HPS and the PMM assays (0.383, 0.360 and 0,225 respectively).

Table 4: Correlation matrix between phenolic contents and antioxidant activities

	PMM	FIC	HPS	PAC	FC
PMM	1				
FIC	0.05	1			
HPS	0.87*	0.06	1		
PAC	0.225	0.383	0.360	1	
FC	0.188	0.057	0.052	0.005	1

Abbreviations: PMM: phosphomolybdate method. FIC: Ferrous ion chelating, capacity. HPS: Hydrogen peroxide scavenging capacity. PAC: Phenolic acids content. FC: Flavonoids content. *: Significantly different ($p<0.05$).

DISCUSSION

It is difficult to compare our results with historical data. Indeed, the extraction of phenolic compounds from their natural matrix is complicated by their diversity and their susceptibility to oxidation and hydrolysis [12]. Similarly, several factors can influence quantifications such as parameters related to the extraction method (e.g., temperature, time contact, solvent to solid ratio, solvent type) [13], variety, environmental conditions, the mode of conservation substrates extraction, the genetic factors and the degree of fruit ripening [14].

The composition and concentrations of phenolic acids of the orange leaves analyzed were not compared with the bibliographic data. The database invested does not provide published results on the phenolic composition of orange leaves. The present work is an initiation into this line of research. Poncirin and AP7G identified in leaves of sweet and sour oranges were not detected in other work on orange by product. Kim et *al.* [15] have found poncirin in peels of mandarin (*C. unshiu*) nevertheless, this flavanone was not quantified. According to Menichini et *al.* [16], apigenin is detected in the leaves of *C. medica* L. cv. Diamante (Diamante citron) but it is not detected in the peels.

Based on the reducing power of leaves, the varieties are classified as following: (Bigarade-Portugaise) > Thomson > (Washington-Sanguinelli) > Double fine > Jaffa. The order of antioxidant capacity based on hydrogen peroxide scavenging capacity of leaves extracts in terms of relative antioxidant activity as follows: Bigarade>(Portugaise +Thomson)> Washington>Sanguinelli> (Double fine+Jaffa). According to the data, it can be seen that whatever the variety and the considered part of the plant is, the EDTA which proves the most FIC efficiency.

L'EDTA, ligand hexadenate dont sa concentration de liaison avec le fer est de l'ordre de 4.8×10^{8} M^{-1}, est considéré comme un excellent agent de chélation, sa capacité de se lier aux ions de métaux lourd peut être employée pour séquestrer les oligo-métaux [17]. Ferrous ion, commonly found in food systems, is well known as an effective pro-oxidant [18]. Polyphenols can chelate pro-oxidant metal ions, such as iron and copper, thus preventing free radical formation from these pro-oxidants [19]. Flavonoids were known to retain free radical scavenging capacity by forming complexes with metal ions [20].

A weak correlation observed between the phenolic acids contents of leaves and the FIC, HPS and the PMM assays, reflecting the moderate contribution of phenolic acids to the antioxidant capacities of orange leaves. These results corroborate with those of Zilic et *al*. [21] who reported a negative correlation between some phenolic acids such as ferulic acid and antioxidant capacity in durum wheat, determined by DPPH radical scavenging.The findings of this study indicate that antioxidant activities increased proportionally to the polyphenol content. Thus, the leaves of Bigarade present the highest levels of total phenols and present the most pronounced antioxidant activities followed by the leaves of Thomson and Portugaise. The lowest values are assigned to Jaffa variety which presents the lowest content in polyphenols. In contrast, flavonoids and phenolic contents do not show a contribution in antioxidant activities. The spider diagram is used to better visualize this relationship (Fig. 1).

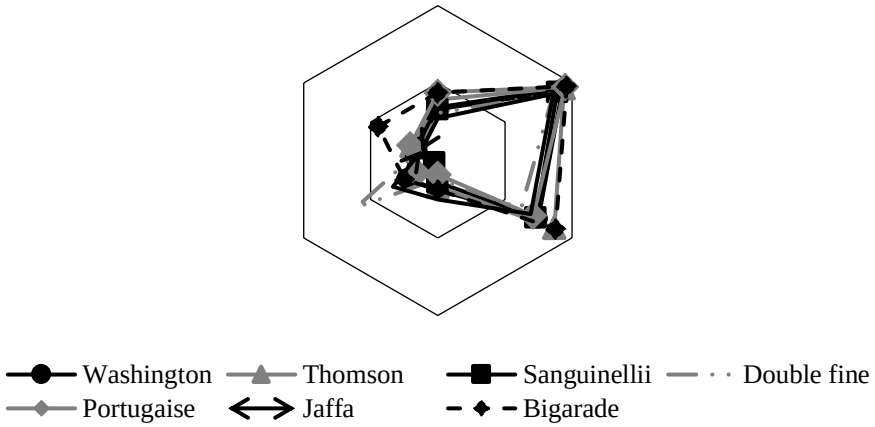

Figure 1: Comparison of antioxidant activity, total phenolic, phenolic acids and flavonoids contents of orange leaves.
FC: flavonoids content. PAC: phenolic acids content. TPC: Total phenolic content. PMM: phosphomolybdate method. FIC: Ferrous ion chelating capacity. HPS: Hydrogen peroxide scavenging capacity.

CONCLUSION

In this work we have proposed the characterization of the distinctive phenolic compounds from the leaves of sweet and sour orange varieties. The antioxidant activity of the phenolic extracts of leaves from the same varieties was assessed using three methods (PMM, HSP and FIC).

Two phenolic acids (gallic and ferulic acids), one flavones (AP7G), two flavonols (kaempferol and rutin) and four flavanons glycosids (narirutin, naringin, hesperidin and poncirin) were characterized in the first time by HPLC-DAD. The quantification of the identified flavonoids has shown that the Kaempferol was the major flavonoids in *C. sinensis* L. However, apigenin-7-glycoside and rutin constituted the greater part of total flavonoids in the leaves of *C. aurantium* L, respectively. The data indicate also that Bigarade, Thomson and Portugaise varieties showed strong antioxidant activities ((Absorbances: 0.39 to 0.44) PMM, HPS (95 to 96%) and FIC (71 to 88%), respectively) when compared with other tested varieties (Washington Navel, Sanguinelli, Double fine and Jaffa), a fact which encourages the prospect of its recovery as a source of powerful natural antioxidants. This information will probably be useful for the utilization of orange leaves as antioxidants in food and drug preparation.

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