Phenolic compounds from Citrus leaves: Antioxidant activity and enzymatic browning inhibition

Article *in* Journal of Complementary and Integrative Medicine · January 2017

DOI: 10.1515/jcim-2016-0030

CITATIONS
6

READS
1,201

6 authors, including:

Bachra Khettal
Ethnobotany and Plants Biotechnology Laboratory,
28 PUBLICATIONS **73** CITATIONS
SEE PROFILE

Nabil Kadri
Université de Béjaïa
41 PUBLICATIONS **96** CITATIONS
SEE PROFILE

Ahmed Adjebli
Université de Béjaïa
5 PUBLICATIONS **23** CITATIONS
SEE PROFILE

Farid Dahmoune
Université de Bouira
80 PUBLICATIONS **734** CITATIONS
SEE PROFILE

Bachra Khettal[1] / Nabil Kadri[2,3] / Karim Tighilet[1,2] / Ahmed Adjebli[4] / Farid Dahmoune[2,3] / Fadila Maiza-Benabdeslam[5]

Phenolic compounds from *Citrus* leaves: antioxidant activity and enzymatic browning inhibition

[1] Laboratoire de Biotechnologie végétale et Ethnobotanique, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria, E-mail: bachra_khettal@yahoo.fr
[2] Département de Biologie, Faculté des Sciences de la Nature et de la Vie et Sciences de la Terre, Université de Bouira, Bouira, Algeria
[3] Laboratoire de Biochimie, Biophysique, Biomathématique et Scientometrie (L3BS), Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algeria
[4] Laboratoire d'Ecologie Microbienne, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algeria
[5] Laboratoire de Biotechnologie végétale et Ethnobotanique, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algeria

Abstract:
Background: Phenolic compounds from *Citrus* are known to be a topic of many studies due to their biological properties including antioxidant activity.
Methods: Methanolic and aqueous extracts were isolated from *Citrus* leaves of different species (*C. clementina*, *C. limon*, *C. hamlin*, *C. navel*, *C. aurantifolia*, *C. aurantium* and *C. grandis*) harvested in Algeria.
Results: The results showed that aqueous extracts of all species are rich in total phenolic compounds and flavonoids (from 68.23 to 125.28 mg GAE/g DM) and (from 11.99 to 46.25 mg QE/g DM) respectively. The methanolic and aqueous extracts were examined for *in vitro* antioxidant properties using various antioxidant assays. For aqueous extracts, *C. limon* showed an important DPPH radical scavenging activity (IC_{50} 35.35 µg/mL), and *C. clementina* exerted the highest ABTS radical scavenging activity (1,174.43 µM ET/g DM) and a significant ferric reducing potential (30.60 mg BHAE/g DM). For methanolic extracts, *C. clementina* showed the highest antioxidant activity for all the realized assays (IC_{50} 41.85 µg/mL, 378.63 µM ET/g DM and 13.85 mg BHAE/g DM) for DPPH, ABTS radicals scavenging activities and ferric reducing potential respectively. Antiperoxidase and antipolyphenol oxidase activities of these samples were also evaluated.
Conclusions: In this investigation, the assessment of antiperoxidase activity proved that the leaves extracts of different species were able to inhibit peroxidase activity. However, this inhibition varied with the species and the source of these enzymes. On the other hand, the aqueous extracts of different species showed moderate inhibition of polyphenol oxidase, while no effect on these enzymes was obtained with methanolic extracts.

Keywords: antioxidant activity, *Citrus*, peroxidase, phenolic compounds, polyphenol oxidase
DOI: 10.1515/jcim-2016-0030
Received: April 12, 2016; **Accepted:** August 15, 2016

Introduction

Oxygen is an essential element for our survival and our development, but when its metabolism is deregulated, it generates radical species which are defined as molecules having one or more unpaired electrons in their outer layers [1]. These free radicals react with the tissues causing oxidative damage. For that, our body is equipped with a complex system of antioxidant defense, endogenous enzymes (SOD, catalase and glutathione peroxidase), not exogenous enzymes. However, under certain conditions, an imbalance caused by an excessive production of free radicals or by a decrease in antioxidant defense called oxidative stress often cause of molecular alterations (proteins, lipids, DNA and carbohydrates) participating in the development of many diseases such as atherosclerosis, neurodegenerative diseases, cardiovascular diseases, etc. [2]. Furthermore, the use of polyphenols as antioxidants may reduce the risk of diseases called oxidative damage [3]. For a long time, people

Bachra Khettal is the corresponding author.

have been enjoying the soothing properties and analgesic of medicinal plants. Today two-thirds of the pharmacopoeia are using their healing properties, they are largely composed of phenolic compounds such as phenolic acids, flavonoids and tannins [4].

On the other hand, the consumption of food during browning (fruits and vegetables) can cause oxidative stress therefore health damage. This phenomenon can also cause not negligible economic losses. It consists of a coloring change during the aging of fresh food or during the implementation of the various conservation or manufacturing processes; it results from deterioration during mechanical, technological or natural treatments [5]. These deteriorations frequently harm to the organoleptic and food nutritional qualities, thus leading to considerable economic losses and health problems during consumption [6]. The enzymatic browning is the result of the transformation by the intermediary of a specific enzymatic system of phenolic colored polymer compounds (melamine). Reaction of initiation being an oxidation of the phenolic compounds by polyphenol oxidase (PPO) and peroxidase (POD) [5]. PPO is probably present in all plants. It belongs to the group of the oxidoreductases with two copper atoms. It catalyzes the oxidation of the phenolic compounds in the presence of molecular oxygen as a co-substrate [7]. This enzyme catalyzes two distinct reactions: the hydroxylation of the monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones, which lead to the formation of black or brown pigments [8]. PODs of plants, also called PODs of class III, catalyze the oxidation of several substrates whose phenols in the presence of hydrogen peroxide (H_2O_2) are implied in several physiological processes related to growth and resistance to biotic and abiotic constraints. The diversity of their quantitative and qualitative aspects made these oxidoreductases potential markers for several plants [9].

Many works were devoted to the search of inhibitors of the enzymatic browning and several techniques and mechanisms were developed in order to maintain quality, to prolong the shelf life of crop product and for enzyme classification. The inhibition of these enzymes in the fruits and vegetables is generally carried out by using physical treatments (bleaching, congelation, lowering of the pH) or by addition of the chemical additives (sulfites, ascorbic acid, polyvinylpolypyrrolidone [PVPP]) [10]. Besides, in spite of the multitude of the physico-chemical treatments acting against the enzymatic browning, no efficient substitute, cheap, practical and without adverse effects on the organoleptic properties of food products have been able to serve as an alternative to this product. Furthermore, the current request of the consumers turn more and more to a natural food deprived of synthetic additives [11].

Citrus leaves are selected based on their local use in traditional medicine in the treatment of many diseases and for their nutritional virtues. It is an important crop with world production estimated at 115 million tons per year. From 2010 to 2011, 571 thousand tons were produced in Algeria which is the 19th producer in the world and the 3rd in the Arab Maghreb Union [12]. *Citrus* leaves are an important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoid and phenolic compounds that have assumed great importance, and recently proposed the use of antioxidant vegetable extracts both as an alternative to food preservation technology and as prophylactic agents for some human diseases [13, 14].

The objectives of this study were to determine and to compare the content of phenolic compounds and the antioxidant capacity of leaves from seven selected *Citrus* species and to investigate the capacity of these phenolic compounds to reduce both POD and PPO activities produced by organic methods from five leafy vegetable extracts.

Materials and methods

Chemical reagents

All chemicals were purchased from Sigma-Aldrich (Germany).

Sample preparation

The leaves of six species of the genus *Citrus*: *C. clementina*, *C. aurantium*, *C. limon*, *C. sinensis*: vr *Thomson navel* and *hamlin*, *C. grandis osbeck*, and *C. aurantifolia* used in this study were harvested from different regions of Bejaia (INRAA, Amizour and Akbou, North East of Algeria) during January 2013. They were cleaned with running water, freed of dust and then shadow dried in the shade for 7 days and then transferred to an oven at 40 °C, until a stable weight. The dried plant materials were ground to granulometry lower than 250 mm [15].

Extraction of phenolic compounds

Twenty grams of each powder factory were extracted by absolute methanol at the ambient temperature (25 °C) for 24 h (200 mL, w/v: 1/10). The extracts were filtered and evaporated in the drying oven at 40 °C until the complete evaporation of methanol to obtain the crude extracts, and kept in the dark at 4 °C until tested [16]. In other part of the study, a second extraction was realized according to Pérez et al. [17]. Twenty grams of each sample were made to backward flow during 3 h with 60 °C in 200 mL of distilled water. After maceration the obtained extracts were filtered and freeze-dried using a lyophilizer of mark "CRIST".

Determination of total phenolic content

The amount of total phenolic compounds was determined by the Folin–Ciocalteu reagent using the method described by Coe et al. [18]; 1.5 mL of Folin–Ciocalteu reagent (0.1N) was added to 200 µL solution of both methanolic and aqueous extract. The solution was mixed and incubated for 5 min at the darkness; 1.5 mL of a sodium carbonate solution (6 %) was added in the reaction medium. The absorbance was measured at 765 nm after 2 h of incubation at ambient temperature by a SPECORD 50 UV-VIS spectrophotometer. The results were expressed as milligrams of gallic acid equivalent (EAG) per gram of dry matter (mg EAG/g DM).

Determination of flavonoid content

The flavonoid content in the extracts was determined by colorimetric aluminum chloride method described by Kadri et al. [19]; 1 mL of aluminum trichloride (2 %) was mixed with 1 mL of extracts. The absorbance of the reaction mixture was measured at 415 nm after 10 min of incubation to the darkness and ambient temperature against a blank prepared under the same conditions. The concentration of the flavonoids was expressed as milligram of quercetin equivalent per gram of dry matter (mg EQ/g DM).

Determination of total antioxidant activity

DPPH radical-scavenging activity

The test of DPPH (2,2-diphenyl-1-picrylhydrazyl) is based on the trapping of stable DPPH free radical by an anti-radicalizing molecule which involves the reduction of violet-colored DPPH radical to yellow-colored one [20]. The ability of the extract to trap the DPPH radical was determined according to the method of Masuda et al. [21]; 50 µL of DPPH solution (5 mM) was added to 2.45 mL of each extracts of *Citrus* leaves at different concentrations (25–400 µg/mL). In parallel, a negative control was prepared by mixing 2.45 mL of methanol or distilled water with 50 µL of DPPH solution. The absorbance at 517 nm is made after 30 min of incubation to the darkness and ambient temperature. The positive control was represented by a solution of two standards: gallic acid and quercetin. The percentage of DPPH radical reduction is calculated as follow:

$$\text{Radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the sample), A_{sample} is the absorbance of the extract with the DPPH solution. The experiment was carried out in triplicate and the results are mean values.

ABTS assay

ABTS test is based on the capacity of an antioxidant to stabilize an ABTS cation radical (acid 2,2-azinobis (3-éthylbenzothiazoline)-6-sulfonic). In reaction medium, ABTS radical cation of intense color blue green, generated by the oxidation of the ABTS with a strong salt such as the persulfate of potassium which reacted with a donor of hydrogen [22]. The radical scavenging activity of extracts was evaluated by the trapping of ABTS radical according to method of Van den Berg et al. [23].

The ABTS radical generated by mixing an ABTS solution (7 mM) with 2.5 mM of persulfate of potassium ($K_2O_8S_2$) in the dark room for 16 h. Before usage, the ABTS solution was diluted to get an absorbance of 0.7 at

734 nm. 1.9 mL of an ABTS solution was added with 100 µL of extract solution to different concentrations. After 5 min of incubation to the darkness and ambient temperature, the absorbance was measured at 734 nm. Positive control is represented by a solution of a standard antioxidant (Trolox]. The antioxidant capacity by ABTS test was expressed in TEAC (Trolox equivalent antioxidant capacity). The results are given in µM or mM of Trolox equivalent per gram of dry matter. The percentage of scavenger activity of radical ABTS is calculated as follow:

$$\text{Radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the sample), A_{sample} is the absorbance of the extract with the ABTS solution. The experiment was carried out in triplicate and the results are mean values.

FRAP (ferric reducing/antioxidant power) assay

The reducing power of the dried *Citrus* leaves was estimated according to the method described by Amarowicz et al. [24]; 1 mL of the extract to various concentrations was mixed with 2.5 mL of buffer phosphates (0.2 M, pH=6.6) and 2.5 mL of potassium ferricyanide solution $K_3Fe(CN)_6$ (1 %). The mixture was incubated at 50 °C during 20 min; 2.5 mL of trichloroacetic acid (10 %) was added to stop the reaction, then centrifuged at 3000 rpm for 10 min; 2.5 mL of supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution ($FeCl_3$ $6H_2O$) at 0.1 %. The absorbance was measured at 700 nm against a blank prepared under the same conditions. Positive control is represented by a solution of a standard antioxidant butyl-hydroxyanisole (BHA).

Measurement of anti-browning enzyme activity

Crude vegetables extracts

Red cabbage, green cabbage, cardon, spinach and lettuce were used as raw materials for POD and PPO. These cultivars were harvested in the region of Bejaia in North east of Algeria. The extractions of the enzymes were carried out according to the protocol described by Ponce et al. [25]. The vegetables used in this study were cleaned, grounded and then homogenized using a high speed blender for 3 min with 30 mL of distilled water at 4 °C. Filtration through a cloth and a centrifugation at 1000g for 15 minutes at 4 °C were performed. The supernatant, which contained POD and PPO activity, was used as the enzyme source for the experiment.

Determination of POD activity

POD activity was measured in the presence and absence of both methanolic and aqueous extracts from *Citrus* leaves using guaiacol as the substrate and hydrogen peroxide (H_2O_2) as the hydrogen donor; 2.87 mL of the substrate solution (composed of 10 mL of 1 % guaiacol, 10 mL of 0.3 % hydrogen peroxide and 100 mL of sodium phosphate buffer (0.05 M, pH 6.5), 0.03 mL of extracts and 0.1 mL of crude extracts were added. In order to use the right levels of enzymatic activity, the assay volume was adjusted to an adequate dilution to ensure linearity of the assay. As POD activity assay using guaiacol as a substrate is very sensitive and rapid, it is important to use the right levels of enzymatic activity in the extract. For cabbage and cardon, the POD activity was measured at different dilution ratios. Finally, a dilution ratio of 15 µL/mL (v/v) and 65 µL/mL was selected for red and green cabbage respectively, a dilution ratio of 150 µL/mL (v/v) for cardon. For spinach and lettuce, enzymatic crude extracts (without dilution) were used. The enzymatic activity was followed by measuring the change in absorbance at 470 nm for 5 min. For each enzyme source, a reagent blank was prepared with 0.03 mL of distilled water instead of antioxidant. Each antioxidant concentration was tested for each extract in triplicate on four independent lots [25].

Determination of PPO activity

The PPO activity was performed according to the protocol described by Lee et al. [26]. The substrate used is pyrocatechol. The oxidation of pyrocatechol results in the appearance of red-brown color; 1 mL of extracts

was mixed with 0.1 mL of the enzyme extract and 0.9 mL of phosphate buffer (50 mM, pH 6.8). The solution was incubated for 5 min at 25 °C and then added to 1 mL of pyrocatechol (0.2 M). The enzymatic activity was followed by measuring the change in absorbance at 420 nm for 1 min. Each antioxidant concentration was tested for each extract in duplicate on four independent lots. The rates of inhibition of the enzymes are expressed as a percentage calculated as follow:

$$\text{Inhibition rate}\ (\%) = \frac{\text{Initial activity} * - \text{Residual activity} * *}{\text{Initial activity}} \times 100$$

*Enzymatic activity (ΔDO/min) measured in the absence of *Citrus* extract.
**Enzymatic activity (ΔDO/min) measured in the presence of *Citrus* extract.

Statistical analysis

All data in this study are expressed as mean±standard deviation (SD) from individual experiments. The statistical significance was analyzed using the one way analysis of variance (ANOVA) with the STATISTICA software '99 Edition. p-Values less than 0.05 were considered to be significant.

Results and discussion

Total phenolics and flavonoids

The result of phytochemical screening revealed that phenolic compounds constitute the major components of the most active fractions. The Folin–Ciocalteu's assay is one of the oldest methods designed to determine the total contents of phenolics in foods or medicinal plants [27]. The total phenolic and flavonoid compounds in the methanolic and aqueous extracts of *Citrus* leaves varied significantly (*$p<0.05$) from one species to another depending on the variety of *Citrus* and the nature of the used solvent for extraction (Table 1). These results showed that the total phenolic content of the aqueous extracts is higher than that of the methanolic extracts (from 2.477 mg GAE/g DM to 11.668 mg GAE/g DM for the methanolic extracts and from 68.23 mg GAE/g to 125.287 mg EAG/g DM in the aqueous extract). The same results were observed for the flavonoid content (1.04 mg QE/g DM to 7.99 mg QE/g DM for the methanolic extract and from 11.99 mg QE/g DM to 46.25 mg QE/g DM for the aqueous extract). High levels of total phenolic and flavonoid content were detected in the aqueous extract of *C. clementina* (125.287 mg GAE/g DM and 46.25 mg QE/g DM respectively).

Table 1: Total phenolic and total flavonoid contents of *Citrus* leaves.

Extracts	Total phenolic content, mg GAE/g DM	Flavonoids, mg QE/g DM
Aqueous extract		
C. clementina	125.28±4.15^{a}	46.25±2.58^{a}
C. aurantifolia	106.05±2.73^{b}	38.36±1.47^{b}
C. limon	98.06±3.01^{b}	38.73±1.54^{b}
C. navel	86.48±7.94^{c}	19.78±1.98^{c}
C. hamlin	84.24±7.33^{c}	18.28±2.75^{c}
C. aurantium	69.97±1.67^{d}	11.99±1.80^{d}
C. grandis	68.23±4.32^{d}	13.06±1.78^{d}
Methanolic extract		
C. clementina	11.67±0.82^{a}	7.99±0.42^{a}
C. aurantifolia	5.77±0.16^{c}	2.72±0.27^{d}
C. limon	3.83±0.78^{d}	2.83±0.36^{d}
C. navel	6.99±0.60^{b}	4.20±0.35^{c}
C. hamlin	4.49±0.19^{d}	2.72±0.40^{d}
C. aurantium	7.77±0.38^{b}	5.08±0.40^{b}
C. grandis	2.48±0.07^{e}	1.04±0.18^{e}

Values are mean±standard deviation (n=3). Means followed by the same letter are not different according to ANOVA Analysis of variance (as in Table 1). GAE, gallic acid equivalents; QE, quercetin equivalents.

These results are in agreement with those reported by Muthiah et al. [28], which showed that the content of total phenolic compounds in the extracts of *C. aurantium* and *C. limon* varies between 7.39 and 33.05 mg/g and with those found by Tawaha et al. [29], which showed that the rates of the phenolic compounds of the aqueous extracts of two varieties of *C. sinensis* (*Thomson navel* et *hamlin*) are very close (23.4 mg GAE/g). According to our results, the average of the polyphenol rates in methanolic extracts of various varieties of *Citrus* was approximately 6 mg GAE/g. This concentration is comparable with that of the phenolic compounds of the methanolic extracts of other *Citrus* species like *Citrus medica* vr. *diamante* [30] and *C. sinensis* [31]. The obtained results concerning proportions of flavonoids in total phenolic compounds are in agreement with those found by Bougandoura and Bendimerad [32], which deduced that the flavonoids account 43.24 % of total phenolics in the methanolic extracts and does not exceed 24.84 % in the aqueous extracts.

Although the results suppose that the methanolic extracts were flavonoids. Another investigation reported by Muthiah et al. [28] suggests that the proportions of the flavonoids based on the total polyphenolic depend both on the species of used *Citrus* and part of plant. Indeed, Menichini et al. [30] have shown that flavonoid content of *C. medica* is lower than total phenolic in methanol extracts. On the other hand, the assays of total phenolic in the extracts of *C. limon*, *C. aurantium* and *C. limetta* showed that the rate of flavonoids varies according to the part of the plant used (from 0.51 to 21.62 mg EQ/g). The concentrations were classified in decreasing order: *C. limetta* (zest)> *C. aurantium* (zest)> *C. limon* (zest)> *C. aurantium* (leaves)> *C. limetta* (leaves)> *C. limon* (leaves)> *C. limetta* (fruit)> *C. aurantium* (fruit)> *C. limon* (fruit) [28].

Several factors can affect the rate of phenolic and flavonoid content such as the presence of some chemical groups (ascorbic acid, organic acids, sugars, aromatic amines) which can also react with the Folin–Ciocalteu reagent [33], but also the period of the crop and the type of the species [34], method of conservation of mining substrates and degrees of leaf maturation [35].

Total antioxidant activity

DPPH radical-scavenging activity

Many studies illustrate the importance given to natural antioxidants in the fields of food processing and medical industry, but also their protective roles against the oxygen reactive species and the correlation between bioactive compounds of plant materials and their antioxidant capacity [36]. The obtained results showed that the different species of *Citrus* leave extracts present a DPPH scavenging activity with percentages of 48 % and 84 % at a concentration of 0.1 mg/mL (Table 2). This scavenging activity remains lower than that obtained with gallic acid or quercetin. These two pure phenols were used as a reference to have a DPPH scavenging activity over 90 % at a concentration of 0.01 mg/mL. No significant difference of the DPPH radical scavenging power has been observed between methanolic and aqueous extracts of *C. naval, C. grandis, C. clementina* ($p>0.05$). For *C. aurantifolia*, *C. hamlin* and. *C. limon*, the aqueous extracts are slightly more effective than methanolic extracts. The highest scavenging effect was observed for the aqueous extract of *C. limon* (IC_{50} 35.35 µg/mL) while the lowest activity was found in methanol extract of *C. hamlin* (IC_{50} 95.78 µg/mL). These results are in accordance with those found by Muthiah et al. [28], for *C. limetta* and *C. aurantium* (IC_{50} 0.148 mg/mL and 0.142 mg/mL respectively) and by Jabri-karoui and Marzouk [37], for *C. aurantium* (IC_{50}= 0.190 mg/mL) but significantly better than the methanolic leaf extract of *C. medica* vr. *diamante* (IC_{50}=0.5 mg/mL) [30].

Table 2: Antioxidant activities of some *Citrus* leaves.

Extracts	DPPH IC_{50}, µg/mL	ABTS, µM ET/g DM	Reducing power, mg BHAE/g DM
Aqueous extract			
C. clementina	43.40±2.47^{e}	1,174.43±1.60^{a}	30.60±0.44^{a}
C. aurantifolia	65.42±2.60^{d}	714.58±9.97^{c}	28.86±0.26^{b}
C. limon	35.35±1.45^{f}	874.54±1.20^{b}	26.03±1.96^{c}
C. navel	82.36±0.94^{b}	528.07±2.68^{e}	22.12±0.61^{e}
C. hamlin	74.99±1.50^{c}	666.20±11.69^{d}	26.23±2.47^{c}
C. aurantium	72.44±1.36^{c}	727.63±9.06^{c}	18.99±1.76^{f}
C. grandis	91.04±1.47^{a}	491.63±14.89^{f}	24.37±1.24^{d}
Methanolic extract			
C. clementina	41.85±1.75^{d}	378.63±5.81^{a}	13.85±0.23^{a}
C. aurantifolia	95.32±2.06^{a}	241.23±1.84^{d}	10.86±1.53^{b}
C. limon	78.23±1.57^{b}	169.22±0.91^{e}	09.87±0.24^{c}
C. navel	80.58±1.88^{b}	260.63±9.73^{c}	10.84±0.18^{b}

C. hamlin	95.78±0.82[a]	149.83±5.03[f]	05.99±0.20[e]
C. aurantium	68.44±3.71[c]	353.48±3.81[b]	13.34±0.11[a]
C. grandis	94.63±1.26[a]	167.59±3.14[e]	07.19±0.18[d]

Values are mean±standard deviation (n=3). Means followed by the same letter are not different according to ANOVA Analysis of variance. ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; DPPH, 2,2-diphenyl picrylhydrazyl; IC_{50}, effective concentration 50 %; TE, Trolox equivalents; BHAE, butyl-hydroxyanisole equivalents.

For the same plant species, the variability of the results of DPPH scavenging activity can be explained not only by the nature of the used plant part and its degree of maturation but also by the applied extraction method [38]. The influence of the extraction solvent on the results of DPPH test was shown. The radical scavenging activity of DPPH of methanolic extracts of *C. aurantium* flowers reported by Karimi et al. [39] (IC_{50}=300 μg/mL) is significantly lower than that we found for the methanol extracts of the leaves (IC_{50}=68 μg/mL).

The difference in the DPPH reducing power of the extracts of different *Citrus* species is probably due to the composition variability of antioxidant metabolites of these species mainly the phenolic compounds such as ascorbic acid, tocopherol, flavonoids and tannins [40]. Polyphenols and flavonoids are good electron donor and/or hydrogen, and their antioxidant power varies from one compound to another [41, 42].

ABTS assay

In order to support the antioxidant efficiency of our extracts, the ABTS assay was used with comparison with natural antioxidants (α-tocopherol, flavonoids and flavones, carotenoids and L-ascorbic acid) [43], or synthetic as 3,5-di-tertiarybutyl-4-hydroxytoluene (BHT); 3-tertiarybutyl-4-hydroxyanisole (BHA) or tertiobutylhydroxyquinone (TBHQ), anti-free-radical efficiency was expressed in TEAC where Trolox is a substance identical to the vitamin E (α-tocopherol). The higher the TEAC value, the greater the antioxidant activity is powerful [44].

The results in Table 2 indicate that at a concentration of 0.1 mg/mL, the percentage inhibition of the radical ABTS by the methanol extracts of *Citrus* leaves varies from 149.83 to 378.63 μM TE/g. Instead, for aqueous extracts, they vary from 491.63 to 1,174.43 μM TE/g with significant differences ($p<0.05$) from one species to another and from an extract to another. However, the efficiency of free radical of aqueous extracts is slightly better regardless of the species. The aqueous extracts of *C. clementina* and *C. limon* record the highest anti-radical ABTS activities with concentrations of 1,174.43 and 874 μM TE/g, respectively while the methanolic extract of *C. grandis* has the lowest activity (167.59 μM TE/g). The scavenger ABTS radical activity of *Citrus* extracts is lower than those obtained for the standard antioxidants such as caffeic acid, gallic acid, quercetin, rutin, BHA, BHT and TBHQ. Indeed, it has been reported that they have TEAC values between 2.56 and 22.3 mM Trolox/g [45].

FRAP (ferric reducing/antioxidant power) assay

In addition to the methods previously described, the antioxidant activity was evaluated by the FRAP assay based on the ability of extracts to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The reduction of ferric ions is accompanied by a color change from yellow to green. The intensity of the color depends on the reduction potential of the compounds present in the reaction medium [46]. For the same concentration of aqueous and methanol extracts (0.2 mg/mL), the FRAP activity of the *Citrus* extracts evaluated by the change in absorbance at 700 nm is significantly smaller than the standards.

According to the obtained results (Table 2), the reducing capacities of aqueous extracts of *Citrus* were classified in decreasing order as follows: *C. clementina* *C. aurantifolia* *C. hamlin* *C. limon* *C. grandis* *C. navel* *C. aurantium*. While the reducing capacities of methanolic extracts of *Citrus* were classified in decreasing order as followings: *C. clementina* *C. aurantium* *C. aurantifolia* *C. navel* *C. limon* *C. grandis* *C. hamlin*. With a significant difference ($p<0.05$) from one species to another and from one extract to another. Our results are in agreement with previous study of Lagha-Benamrouche and Madani [47] indicate that the methanolic extracts of the *C. aurantium* leaves were provided with a higher reducing power than that of the leaves of some varieties of sweet orange (Thomson navel, Washington, Portuguese, …). These results also confirm what has been advanced by other studies stating that the reduction potential is due to the nature of antioxidants present in each extract [39].

Relationship between the total antioxidant capacity and the total phenolic and flavonoid content

In order to evaluate if the antioxidant activity by the DPPH test of our various *Citrus* extracts depends on their levels of phenolic compounds, a relationship was assessed, by linear regression first, between power scavenging DPPH (expressed as IC_{50}) and the amount of total phenolic (expressed as mg gallic acid EQ/g DM) and second between power scavenging DPPH (expressed by IC_{50}) and flavonoid rate (expressed in mg quercetin EQ/g DM) (Table 3). The obtained results showed that the *Citrus* present a high correlation between their efficiencies to trap the DPPH radical and the rate of total phenolic and flavonoid content of their methanolic (r=0.872 and r=0.936 respectively) and aqueous (r=0.820 and r=0.914, respectively) extracts (Table 3). However, this correlation is slightly higher when the extraction is carried out with methanol. Our results are in accordance with those found by Floegel et al. [48].

Table 3: Correlation matrix between antioxidant and antioxidant activities.

Extracts	TPC	FLA	DPPH	ABTS	PR
Aqueous extract					
TPC	1				
FLA	0.956	1			
DPPH	0.892	0.914	1		
ABTS	0.811	0.795	0.796	1	
RP	0.863	0.846	0.714	0.625	1
Methanolic extract					
TPC	1				
FLA	0.939	1			
DPPH	0.871	0.952	1		
ABTS	0.921	0.847	0.796	1	
RP	0.834	0.761	0.606	0.93	1

TPC, total phenolic compounds; FLA, flavonoids; RP, reducing power; ABTS, antiradical activity with ABTS; DPPH, antioxidant activity with DPPH.

The correlation analysis has allowed to deduce that the polyphenols in the extracts of *Citrus* leaves are responsible for up to 87 % of the anti-radical ability of DPPH regardless of the used extraction solvent. Conversely, by comparing the coefficients of obtained correlation, we deduce that the flavonoids are responsible for the majority of the DPPH radical scavenging effect than other phenolic compounds which may be present in the case of our extracts such as phenolic acids or even tannins. So, it appears that the antioxidant capacity of *Citrus* species is related to the concentration of polyphenols and more particularly to those of flavonoids.

The relationship between the total polyphenol content and flavonoids and the ABTS radical activity is illustrated in Table 3. The correlation coefficients for the polyphenols (r=0.921 for the methanolic extracts and r=0.811 for aqueous extracts) or flavonoids (r=0.888 for the methanolic extracts and r=0.795 for the aqueous extracts) showed that the degree of dependence between the phenolic compounds and the antioxidant power of the *Citrus* extracts is very high for both the aqueous extract and the methanolic extracts, so it appears clearly that these are the flavonoids that play a leading role in antioxidant activity.

Various investigations showing the involvement of phenolic compounds in particular flavonoids in the radical scavenger activity have been reported. Floegel et al. [48] indicated the existence of a positive correlation between the activity of scavenging ABTS radical cation and phenolic content (r=0.946), 72 % of the scavenging activity is performed by flavonoids.

Although polyphenols are compounds which carry a majority of the antioxidant activity, it is important to note that antioxidant activity carried out can be attributed or influenced by non-phenolic compounds such as lipids, sugars and chlorophyll. The study conducted by Heim et al. [49] showed that the hydroxyl groups of the phenolic and flavonoid compounds play an important role in the antioxidant power. While the works of Naczk and Shahidi [42] showed that the flavonoid aglycones are more active than glycosylated forms. On the other hand, Zhang and Wang [50] reported that the antioxidant activity of the phenolic compounds is mainly due to the number of hydroxyl groups and their positions on the aromatic ring.

A positive correlation between the reducing power (expressed in equivalent mg BHA per grams of dry matter) and the amount of phenolic compounds was obtained (Table 3). Based on this analysis, the reducing power of the aqueous or organic extracts is due to ≈83 % of polyphenols, mainly flavonoids (r=0.82).

Hayes et al. [51] observed the same trends of correlation between anti-radical power and reducing power. According to Singh and Rajini [52], the correlation is due to the existence of bioactive molecules with a strong reducing and anti-radical properties. Similar results have been reported by several authors showing the exis-

tence of a good correlation between the total polyphenols and antioxidant activities of the plant extracts [48, 53] in particular *Citrus* species extracts [29, 54].

Anti-browning enzyme activity

POD activity

In order to analyze the effects of anti-POD of our *Citrus* extracts, we evaluated the efficacy of both aqueous and methanolic extracts on the POD activity of different sources of leafy vegetables. We observed that the rate of POD activity differs depending on the enzyme source used. So, it appeared that the enzyme extract of cabbage and in particular red cabbage presents the highest rate of POD. These results are similar to those reported by Alikhani et al. [55] and Ponce et al. [25]. The difference in catalytic efficiency of these enzymes of different plants is assigned in general to their affinity for the substrate, in the presence of multiple enzyme forms in the same plant and/or to parameter variations of the reaction medium. According to the results shown in Table 4, the methanolic extracts of different *Citrus* species inhibit POD activity. The inhibition rate varies depending on species of *Citrus* and according to the enzyme source. Thus, whatever the enzyme source for the same concentration (1 mg/mL), the extracts of *C. limon*, *C. clementina*, *C. aurantium*, *C. Hamlin* and *C. aurantifolia* showed inhibition rates of 19, 44 to 60.80 %. However, the inhibitory activities of POD by leaf extracts of *Citrus* remain far lower than those obtained with standard polyphenols (gallic acid and quercetin). Regarding the methanolic extracts of *C. navel* and *C. grandis*, they have no effect on the POD activity of spinach leaf. The best inhibition rate (60.80 %) was recorded for methanolic extract of *C. aurantifolia* on lettuce activity.

Table 4: Effect of *Citrus* leave antioxidant extracts on the relative activity of peroxidase expressed as percentage of reducing activity antienzymatic browning activity.

	Red cabbage (15 µL/mL)	Green cabbage (65 µL/mL)	Cardon (150 µL/mL)	Lettuce (300 µL/mL)	Spinach (300 µL/mL)
Aqueous extract					
C. clementina	53.45	26.00	38.61	18.74	31.85
C. limon	26.43	25.83	28.65	00	29.29
C. navel	08.00	05.06	25.47	00	20.02
C. hamlin	12.14	06.04	16.74	00	06.89
C. aurantifolia	15.94	11.54	25.60	00	00.29
C. aurantium	04.57	04.72	31.05	00	12.01
C. grandis	19.86	02.65	27.57	00	22.17
Methanolic extract					
C. clementina	31.06	27.60	23.96	54.06	22.00
C. limon	50.50	50.63	23.40	53.53	36.63
C. navel	29.56	03.78	17.18	29.93	02.28
C. hamlin	32.02	13.72	04.42	33.79	17.21
C. aurantifolia	24.98	21.13	19.76	60.80	19.49
C. aurantium	20.50	28.85	27.09	47.61	19.44
C. grandis	14.32	10.10	18.52	17.04	00.82
Standards					
Gallic acid	79.64	84.93	79.93	77.22	87.92
Quercetin	88.49	92.31	91.79	88.99	93.05

Values are mean±standard deviation (n=3).

The inhibitory efficiency of the POD was evaluated by determining the IC_{50} "Concentration of inhibitor required to reduce 50 % of the enzyme activity." According to our results (Table 4), it appears that the leave methanolic extracts of the species *C. limon* and *C. clementina* showed the best efficiency to inhibit POD activity, with IC_{50} of 110.28–179.36 µg/mL and 141.67–155.10 µg/mL respectively. The anti-POD efficiency of extracts of these two species of *Citrus* remains lower compared to that of quercetin and gallic acid, which have an IC_{50} of 3.87–10.88 µg/mL respectively.

On the other hand, we observed that the aqueous extracts of different *Citrus* leaves exhibited inhibitory activities of POD extracted from all leafy vegetables (Table 4). For a concentration of 100 µg/mL, the observed inhibition rates vary depending on the *Citrus* species tested and the enzyme source. Except the POD extract of lettuce where only the aqueous extract of *C. clementina* has an inhibiting enzyme activity (18.75 %), POD s

activities of other leafy vegetables were inhibited by almost all of the *Citrus* extract tested with variable rates. Thus, the POD activity was inhibited by cardon aqueous extracts of six species of *Citrus* with inhibition rates ranging from 16.75 % (*C. hamlin*) to 38.61 % (*C. clementina*). The best inhibition rate (53.45 %) was obtained with the extract of *C. clementina* on POD of red cabbage, while lower rates were obtained by extracts of *C. grandis* (2.65 %) and *C. aurantifolia* (0.29 %) on PODs of green cabbage and spinach, respectively. However, these inhibition rates are much lower than those obtained by the phenolic antioxidants, quercetin and gallic acid which are greater than 77 %.

Inhibitory effects of the plant POD were observed for extracts of plants but essentially for their essential oils. Therefore, Ponce et al. [25] showed that the essential oils of *C. limon* inhibit POD activity of cabbage, cardon, spinach and lettuce. The studies conducted by Mousavizadeh et al. [56], Alikhani et al. [55], and Ponce et al. [25] indicated a difference on the inhibition efficiency of the cabbage POD activity of the extracts of different kinds of *Citrus* plants. The inhibition efficiency of the methanolic extracts is better than the aqueous extracts. Indeed, the concentrations required to inhibit 50 % of the POD activity with the aqueous extracts were almost two times higher than those used by the methanolic extracts. The different inhibitory potential of our extracts can be attributed to the presence and the variability in bioactive substances in terms of nature and quantity. On the other hand, whatever the *Citrus* extract type, the inhibition efficiency also depends on the source of the used enzyme extract. This variability of action can be explained by the existence of several forms of enzymes (isoenzymes) in the same plant and in the same tissue, and by difference of their physico-chemical and catalytic properties. The pH and temperature of the reaction media may also influence the specificity of POD in a significant manner [57, 58]. The methanolic and aqueous extracts of *C. clementina* which are competitive inhibitors of the POD probably contain phenolic compounds which are structural analogues of the POD substrate (guaiacol). These compounds bind reversibly to the active site of the enzyme and thus block the access of the substrate to the enzyme site. For the aqueous extract of *C. limon,* it has an incompetitive inhibitory activity in both red and green cabbage POD. The active compounds of the aqueous extract which are responsible have no structural analogy with the substrate and bind to the enzyme previously complexed with the substrate.

PPO activity

PPO activity of both aqueous and methanolic extracts of *Citrus* leaves was tested for five leafy vegetables enzymes (red cabbage, green cabbage, cardon, lettuce and spinach). The results obtained indicate that the methanolic extracts do not show any inhibitory effect of PPO activity even at high concentration (1 mg/mL) regardless of the source of the enzyme. However, a decrease in the activity of these enzymes was observed in the presence of aqueous extracts, it varies depending on the enzyme source and the species of *Citrus* (Table 5). For a concentration of 500 µg/mL of *Citrus* extracts, the most important inhibition rates were widely lower than those of ascorbic acid (73–86.86 %). From the obtained results, the aqueous extracts of six species of *Citrus* inhibited PPO of lettuce with rates of 11.9 % (*C. clementina*) to 22.53 % (*C. aurantium*). Whereas for cardon and green cabbage, the extracts of *Citrus* have little or no inhibitory activity (00–10.29 %). For PPO of red cabbage and spinach, the inhibition rate for almost all extracts of *Citrus* varies from 10 % to 20 % with the highest level of PPO red cabbage inhibition by *C. grandis* extract (30.64 %).

Table 5: Effect of *Citrus* leave antioxidant extracts on the relative activity of polyphenol oxidase expressed as percentage of reducing activity.

Aqueous extract	Red cabbage (15 µL/mL)	Green cabbage (65 µL/mL)	Cardon (150 µL/mL)	Lettuce (300 µL/mL)	Spinach (300 µL/mL)
C. clementina	01.62	10.29	00	11.90	13.67
C. limon	21.73	01.06	04.30	12.66	21.67
C. navel	17.91	03.67	01.83	19.31	14.58
C. hamlin	11.54	01.50	00	19.66	02.26
C. aurantifolia	08.36	09.81	04.15	15.46	21.49
C. aurantium	23.00	05.43	00	22.53	04.94
C. grandis	30.64	08.05	02.22	14.08	00.19
Ascorbic acid	80.27	86.86	85.26	73.30	77.23

Previous studies have demonstrated that the natural antioxidants act as inhibitors of PPO fruits and vegetables. However, the results obtained for *Citrus* extracts are in disagreement with many works which report that other plant extracts have a better inhibitory efficiency of the PPO. Kim et al. [11] showed that the onion extracts decrease the rate of the pear browning with an efficiency of up to 54.1 % by inhibiting PPO activity.

These results were confirmed by Lee et al. [26], who showed that taro PPO was inhibited by onion extracts with rates of 54.8 % to 68.5 %. Other studies have also reported similar effects when using natural extracts of plants on the PPO activity [59, 60]. The difference in inhibition rate from an extract to another and an enzyme source to another can be explained by the presence of multiple enzyme forms (isoenzymes) in the enzyme extract which present different structural and physicochemical properties, and/or by the nature of the active molecules of vegetable inhibitor extracts [61].

Conclusions

The results have shown that regardless of the studied *Citrus* species, the aqueous extract is rich in phenolic antioxidants, mainly flavonoids more than methanolic extracts. A very good linear correlation was observed between the antioxidant activities assessed by the DPPH test, ABTS, FRAP and phenolic content of the aqueous extracts of the analyzed *Citrus* leaves. Furthermore, it has been shown that the majority of the *Citrus* extracts presented an inhibitory effect of PODs extracted from different leafy vegetables. However, only the aqueous extracts of *Citrus* presented an anti-PPO effect which varies according to the plant source and the enzymatic species of the studied *Citrus*.

Acknowledgement

The authors are thankful to the members of the Faculty of Natural Sciences and Life, and the Plant Biotechnology & Ethnobotany laboratory, University of Bejaia, Algeria.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Koechlin-Ramonatsco C. Oxygen, oxidative stress and antioxidant supplementation, or another way for nutrition in respiratory diseases. Nutr Clin Metab. 2006;20:165–177.
2. Baudin B. Stress oxydant et pathologies cardiovasculaires. Mt Cardio. 2006;2:43–52.
3. Hannebelle T, Sahpaz S, Bailleul F. Polyphénols végétaux, sources, utilisation et potentiel dans la lutte contre le stress oxydatif. Phytothérapie. 2004;1:3–6.
4. Iserin P, Massou M, Restellini JP, Moulard F, Zha E, Delarouque R. Encyclopédie des plantes médicinales. 2001;4.
5. Vámos-Vigyázó L. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC Crit Rev Food Sci Nutr. 1981;15:49–127.
6. Lee CY, Whitaker JR. Enzymatic browning and its prevention (ACS Symposium Series). Washington, DC: American Chemical Society; 1995.
7. Marusak CM, Trobaugh NM, Flurkey WH, Inlow JK. Comparative analysis of polyphenol oxidase from plant and fungal species. J Inorg Biochem. 2006;100:108–123.
8. Gawlik-Dziki U, Szymanowska U, Baraniak B. Characterization of polyphenol oxidase from broccoli (Brassica oleracea var. Botrytis italica) florets. Food Chem. 2007;105:1047–1053.
9. Baaziz M, Qacif N, Bendiab K, Aouad A. Les Peroxydases des plantes. Aspect théorique et Applications pratiques. Enzymol Métab. 2006;17–20.
10. Nicoli MC, Elizalde BE, Pitotti A, Lerici CR. Effect of sugars and Maillard reaction products on polyphenol oxidase and peroxidase activity in food. J Food Biochem. 1991;15:169–184.
11. Kim MJ, Kim CY, Park I. Prevention of enzymatic browning of pear by onion extract. Food Chem. 2005;89:181–184.
12. FAO. Statistical Databases. 2012 . Accessed 19 July 2013 Available at:www.FAO.org.
13. Aruoma AO. Extracts as antioxidant prophylactic agents. Inform. 1997;8:1236–1242.
14. Kamran G, Youcef G, Ebrahimzadeh MA. Antioxidant activity, phenol and flavonoid contents of 13 Citrus species peels and tissues. Pak J Pharm Sci. 2009;22:277–281.

15. Ma YQ, Chen JC, Liu DH, Ye XQ. Simultaneous extraction of phenolic com-pounds of Citrus peel extracts: effect of ultrasound. Ultrason Sonochem. 2009;16:57–62.
16. Falleh H, Ksouri R, Chaieb K, Karray-Bouraoui N, Trabelsi N, Boulaaba M. Phenolic composition of Cynara cardunculus L. organs, and their biological activities. C R Biol. 2008;331:372–379.
17. Perez YY, Jimenez-Ferrer E, Alonso D, Botello-Amaro CA, Zamilpa A. Citrus limetta leaves extract antagonizes the hypertensive effect of angiotensin II. J Ethnopharm. 2010;128:611–614.
18. Coe S, Fraser A, Ryan L. Polyphenol bioaccessibility and sugar reducing capacity of black green, and white teas. Int J Food Sci. 2013;2013:1–6.
19. Kadri N, Khettal B, Aid Y, Kherfellah S, Sobhi W, Barragan-Montero V. Some physicochemical characteristics of pinus (Pinus halepensis Mill., Pinus pinea L., Pinus pinaster and Pinus canariensis) seeds from North Algeria, their lipid profiles and volatile contents. Food Chem. 2015;188:184–192.
20. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Technol. 1995;28:25–30.
21. Masuda T, Yonemori S, Oyama Y, Takeda Y, Tanaka T, Andoh T. Evaluation of the antioxidant activity of environmental plants: activity of the leaf extracts from seashore plants. J Agri Food Chem. 1999;47:1749–1754.
22. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem. 1999;274:33419–33425.
23. Van Den Berg R, Haenen GR, Van Den Berg H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem. 1999;66:511–517.
24. Amarowicz R, Pegg R, Rahimi-Moghaddam P, Barl B, Weil J. Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem. 2004;84:551–562.
25. Ponce AG, Del Valle CE, Roura SI. Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. LWT-Food Sci Technol. 2004;37:199–204.
26. Lee MY, Lee MK, Park I. Inhibitory effect of onion extract on polyphenol oxidase and enzymatic browning of taro (Colocasia antiquorum var. esculenta. Food Chem. 2007;10:528–532.
27. Roginsky V, Lissi EA. Review of methods to determine chain-breaking antioxidant activity in food. Food Chem. 2005;92:235–254.
28. Muthiah P, Umamaheswari M, Asokkumar K. In vitro antioxidant activities of leaves, fruits and peel extracts of Citrus. Int J Phytopharm. 2012;2:13–20.
29. Tawaha K, Alali FQ, Gharaibeh M, Mohammad M, El-Elimat T. Antioxidant activity and total phenolic content of selected Jordanian plant species. Food Chem. 2007;104:1372–1378.
30. Menichini F, Loizzo MR, Bonesi M, Conforti F, De Luca D, Statti GA. Phytochemical profile, antioxidant, antiinflammatory and hypoglycemic potential of hydroalcoholic extracts from Citrus medica L. cv Diamante flowers, leaves and fruits at two maturity stages. Food Chem Toxicol. 2011;49:1549–1555.
31. Kammoun BA, Boudhrioua MN, Kechaou N. Moisture sorption isotherms experimental and mathematical investigations of orange (Citrus sinensis) peel and leaves. Food Chem. 2012;132:1728–1735.
32. Bougandoura N, Bendimerad N. Evaluation de l'activité antioxydante des extraits aqueux et méthanoliques de Satureja calamintha ssp. Nepeta (L.) Briq. Revue «Nature & Technologie» B-Sciences Agronomiques et Biologiques. 2012;9:14–19.
33. Abd Ghafar MF, Nagendra M, Prasad K, Weng KK, Ismail A. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from Citrus species. Afr J Biotechnol. 2010;9:326–330.
34. Makkar HPS, Singh B, Dawra RK. Effect of tannins rich leaves of oak (Quercus incana) on various microbial enzyme activities of the bovin rumen. Brit J Nutr. 1988;60:287–296.
35. Sharma RR, Goswami AM, Singh CN, Chhonkar OP, Singh G. Catecholase and cresolase activities and phenolic content in mango (Mangifera indica L.) at panicle initiation. Sci Hortic. 2001;87:147–151.
36. Kong KW, Khoo HE, Prasad KN, Ismail A, Tan CP, Rajab NF. Revealing the power of the natural red pigment lycopene. Molecules. 2010;15:959–987.
37. Jabri-Karoui I, Marzouk B. Characterization of bioactive compounds in Tunisian bitter orange (Citrus aurantium L.) Peel and juice and determination of their antioxidant activities. Biomed Res Int. 2013;2013:1–12.
38. Xu G, Ye X, Liu D, Ma Y, Chen J. Composition and distribution of phenolic acids in Ponkan (Citrus poonensis Hort. ex Tanaka) and Huyou (Citrus paradisi Macf. J Food Compos Anal. 2008;21:382–389. Changshanhuyou) during maturity.
39. Karimi E, Oskoueian E, Hendra R, Oskoueian A, Jaafar HZE. Phenolic compounds, characterization and biological activities of Citrus aurantium bloom. Molecules. 2012;17:1203–1218.
40. De Pooter HL, Schamp N. Comparison of the volatile composition of some Calamintha satureja species. In: Brunk EJ, editors. Progress in essential oil research. Berlin: Walter De Gruyter; 1986.
41. Bourgou S, Ksouri R, Bellila A, Skandrani I, Falleh H, Marzouk B. Phenolic composition and biological activities of Tunisian Nigella sativa L. shoots and & roots. C R Biol. 2008;331:48–55.
42. Naczk M, Shahidi F. Extraction and analysis of phenolics in food. J Chromatogr A. 2004;1054:95–111.
43. Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J, Dominguez H. Natural antioxidants from residual sources. Food Chem. 2001;72:145–171.
44. Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chem. 2006;97:654–660.
45. Olszewska MA, Presler A, Michel P. Profiling of phenolic compounds and antioxidant activity of dry extracts from the selected Sorbus species. Molecules. 2012;17:3093–3113.
46. Zou Y, Lu Y, Wei D. Antioxidant activity of a flavonoid rich extract of Hypericum perforatum L. In vitro. J Agri Food Chem. 2004;52:5032–5039.

47. Lagha-Benamrouche S, Madani K. Phenolic contents and antioxidant activity of orange varieties (Citrus sinensis L. and Citrus aurantium L.) cultivated in Algeria: Peels and leaves. Ind Crops Prod. 2013;50:723–730.
48. Floegel A, Kim D, Chung S, Koo SI, Chun OK. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J Food Compos Anal. 2011;24:1043–1048.
49. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. J Nutr Biochem. 2002;13:572–584.
50. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agri Food Chem. 2001;49:5165–5170.
51. Hayes JE, Allena P, Bruntona N, O'Grady MN, Kerry JP. Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid. Food Chem. 2011;126:948–955.
52. Singh N, Rajini PS. Free radical scavenging activity of an aqueous extract of potato peel. Food Chem. 2004;85:611–616.
53. Calabro ML, Galtieri V, Cutroneo P, Tommasini S, Ficarra P, Ficarra R. Study of the extraction procedure by experimental design and validation of a LC method for determination of flavonoids in Citrus bergamia juice. J Pharmaceut Biomed. 2004;35:349–363.
54. Jayaprakasha GK, Girennavar B, Patil BS. Antioxidant capacity of pummelo and navel oranges: extraction efficiency of solvents in sequence. LWT-Food Sci Technol. 2008;41:376–384.
55. Alikhani M, Sharifani M, Mousavizadeh SJ, Azizi M. The antioxidative activity of some essential oils in reducing peroxidase activity and enzymatic browning in some vegetables. J Agric Sci Nat Resour. 2009;16:203–207.
56. Mousavizadeh SJ, Sedaghathoor S, Khorami H. Essential oils as reducing agents of cabbage peroxidase. Sci Hortic. 2011;128:388–392.
57. Shannon IM, Kay E, Lew JY. Peroxidase isoenzymes from horseradish rots. Isolation and physical properties. J Biol Chem. 1996;241:2166–2172.
58. Lee HC, Klein BP. Evaluation of combined effects of heat treatment and antioxidant on peroxidase activity of crude extract of green peas. Food Chem. 1989;32:151–158.
59. Espin JC, Garcia-Ruiz PA, Tudela J, Varon R, Garcia-Canovas F. Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases. J Agr Food Chem. 1998;46:2968–2975.
60. Jang MS, Sanada A, Ushio H, Tanaka M, Ohshima T. Inhibitory effects of 'Enokitake' mushroom extracts on polyphenol oxidase and prevention of apple browning. LWT-Food Sci Technol. 2002;35:697–702.
61. Shmitz GE, Sullivan ML, Hatfield RD. Three polyphenol oxidases from red clover (Trifolium pratense) differ in enzymatic activities and activation properties. J Agri Food Chem. 2008;56:272–280.