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Physico-Chemical Characteristics and antioxidant Activity of Phenolic Compounds and oil of *Citrus aurantium* Seeds from Northwest Algeria

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Abstract

Sour orange (Citrus aurantium) is a tree of the family of Rutaceae. It is cultivated today in many parts of the world; the fruit, leaves, twigs and flowers have numerous applications in food and perfumery. *Citrus aurantium* used in this study originate from Tlemcen in North East of Algeria.
Our study focused firstly on the physicochemical analysis of seed oil of sour orange namely the determination of indices, fatty acids, vitamin E and polyphenols.
Secondly we evaluated total phenols, flavonoids, condensed tannins of *Citrus aurantium* seeds and their antioxidant activity of using in vitro methods: ferric reducing antioxidant power (FRAP) assay and β-carotene bleaching test.
The seeds oil contents is 38.21%, their fatty major acids were palmitic (26.85 %) and linoleic acid (38.29%). The physicochemical analysis of oil showed index values of density, acid value, ester value, saponification value and refractive index estimated respectively: 0926; 1.212, 190.39, 191.52, 1467.
The quantitative estimation of *Citrus aurantium* seeds showed that content of polyphenol is 2.12 mg GA /g DW which the predominant part is represented by tannins (0.3 mg CE /g DW) followed by the flavonoids (0.076 mg CE /g DW). Our results demonstrate that all extracts have antioxidant capacity. Among these extracts, the ethyl acetate fraction of flavonoids showed the highest value of antioxidant activities for seed which might constitute an important source of natural antioxidants.
Keywords: Citrus aurantium seed; oil; phenolic compounds; antioxydant activity; Ferric reducing; β-carotene..

Introduction

Citrus are recognized as one of the world's major fruit crops. These are produced in many countries all around the world with tropical or subtropical climate, and countries of the Mediterranean region are the major citrus producers [1, 2].The Algerian citrus development program occupies in important place in the new agricultural policy of the country, considering the different pedoclimatic agricultural areas [3]. During 2010/2011, 571 thousand tons were produced in Algeria which is the 19th producers in the world and the 3rd in the Arab Maghreb Union [4]. Citrus fruits are mainly used for dessert, juice and jam production. This food and agro-food processing industry yields considerable amount of waste or by-products such as peels, seeds and pulps which represents 50% of the raw processed fruit [5]. The citrus seeds, commonly considered as agro-industrial waste, are a potential source of oil [6]. Literature revealed that citrus seeds oils are a good source of unsaturated fatty acids (FAs) [7]. Anwar and Naseer [5] reported physicochemical characteristics composition of Pakistan seeds and seed oils of citrus fruits. Ajewole and Adeyeye studied the characterization of Nigerian citrus seed oils [8].

Recently, a number of studies have proposed that some fruits or vegetables by products could be a source of natural antioxidants in order to valorize these wastes [9, 10, 11]. Moreover, citrus pomace has been reported to contain natural antioxidants such as phenolic acids and flavonoids [12, 13]. Lagha-Benamrouche and Madani [14] showed the presence of phenolic compounds in peels and leaves of different varieties, with varying proportions. These antioxidants appear to play a role in the prevention of oxidative stress-related diseases and in the reduction of total mortality associated with diets rich in plant foods, particularly fruits and vegetables [15, 16, 17, 18]. Antioxidant activity of a compound is its ability to resist oxidation. The best known antioxidants are the phenolic compounds, ascorbic acid, the β-carotene and tocopherol. Indeed, most synthetic antioxidants or naturally occurring hydoxyphenolics have groups in their structures and antioxidant properties are attributed in part to the ability of these natural compounds to scavenge free radicals such as hydroxyl radical (OH•) and superoxide (O2•) [19]. Lagha-Benamrouche and Madani [14] reported that among all the varieties of citrus, Bigarade presents the highest capacity to slow the rate of oxidation of linoleic acid and β carotene and the highest antiradical activity.

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This study was carried out on seeds of *Citrus aurantium* (Bigarade) growing in Algeria. The objective was to determine physicochemical characteristics and composition of the seeds and seed oils, to quantify phenolic, flavonoids and tannins contents and to evaluate the antioxidant capacity of citrus seeds.

Material and methods

Plant material

Citrus fruit of sour orange (*Citrus aurantium*) used in this study originate from Fhoul Tlemcen in North East of Algeria (latitude 34 52′41″; longitude 1 18′53″). The fruits were cut into small pieces with a knife and seed were collected manually. The seeds were washed with tap water and dried at ambient temperature in the dark until used.

Oil Extraction

Samples of dried *citrus aurantium* seeds were crushed using a blender. A hundred grams seeds were fed into a soxhlet extractor [20]. Fitted with a 1-L round bottomed flask and a condenser. The extraction was executed for 8 h with 0.50 L of hexane. The solvent was evaporated at 40 C using a rotavapor.

Analysis of Extracted Oil

Physical and Chemical Parameters of Oil

Determinations of density, refractive index, acidity and saponification value of the extracted oil were made following AFNOR [21].

Fatty Acid Composition

The fatty acid composition of the oil samples was determined using gas chromatography (Perkin Elmer, Clarus 500, France) coupled to flame ionization detection (GC-FID). Briefly, about 100 µL of oil were added to 100 µg of internal standard (19:0 20µg/100µL). 1mL NaOH in methanol (40 g/mol) was added, the mixtures were vortexed for 5 s and put at 80 C for 20 min. The same procedure was done with 2 mL of BF3 in methanol. 2 mL of NaCl in methanol at 35 % and 2 mL of hexane were added and the tube was shaken for 5min. After decantation, the upper phase was injected into the GC-FID system for analysis. Carrier gas was helium and the temperature for injection and detection was 200 C. The fatty acid methyl esters were identified by comparison of their retention times with standard mixture [22]

Tocopherol Content

-tocopherol (vitamin E) was determined by reverse phase HPLC in oil [23]. The stationary phase was constituted of greffed silica (C18 column, HP ODS Hypersil C18; 200 mm x 4.6 mm; Lara spiral, maintenance temperature of analytical column, 35 C). The mobile phase was a mixture of methanol/water (98/2, v/v) at a flow rate of 1 ml/min. Vitamin E were extracted by hexane, dried under nitrogen and resuspended in methanol. The extracted vitamin was injected into the HPLC system. The HPLC peaks were detected by a UV detector at 292 nm. Representative chromatograms were obtained by injecting standard solutions.

Preparation of extracts

Phenolic compounds in oil

The phenolic compound extraction procedure was based on the work of Pirisi and Bonoli [24, 25]. Two grams of seed was weighed into a centrifuge tube and 1 mL of hexane and 2 mL of methanol: water (60:40, v/v) were added. This mixture was stirred for 2 min in a vortex apparatus, and the tube was then centrifuged at 5000 rpm for 5 min. The methanol:water layer was separated and the extraction was repeated twice. The extracts were combined and evaporated to dryness at 39 C under reduced pressure. Samples were resuspended in 3 mL of methanol.

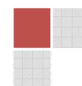
Polyphenols Extraction in Citrus seed

The extraction procedure used was adapted from Yu & Dahlgren [26]. The dried seeds of *Citrus aurantium* (10 g) were ground and extracted with acetone – water (70/30, v/v) by maceration at room temperature for 24 hours. Then the extracts were filtered through whatman filter paper under vacuum. The filtrate was concentrated to dryness under reduced pressure at 45 C and was stored at 4 C, for further investigation.

Flavonoids Extraction in Citrus seed

10g of dried material were extracted with 100 ml of methanol and 5g of carbonate of Calcium by boiling for 1 hour [27]. After filtration through whatman filter paper, the methanol was evaporated under reduced pressure and affording the aqueous extracts. Subsequently, recover the dry extract with 50 ml of boiling water. The aqueous extract was filtred and then fractionated by solvent – solvent extraction, first with diethyl ether, ethyl acetate and then with n-butanol, using a separating funnel (Pyrex). All the fractions were concentrated, dried to constant weight in a vacuum oven at 45 C and kept at 4 C.

Tannins Extraction in Citrus seed



Five grams of powdered material was extracted at 4 C using 200 ml of a mixture of acetone – water (25/45, v/v) for 4 days [28]. The extracts were filtered under vacuum through filter paper and acetone was evaporated under reduced pressure. Subsequently, the dichloromethane (2x 25 ml) was used for the extraction of lipids and pigments from the aqueous extracts using a separating funnel. Afterward, the aqueous phase was extracted with 25 ml of ethyl acetate. This process was repeated 4 times. After filtration, the organic phases (ethyl acetate) containing tannins were recovered and concentrated to dryness under vacuum using a rotary evaporator. The residues obtained after evaporation was stored at 4 C and used for further investigation.

Total phenolic content

Total phenolics content in the citrus seed extract was assayed using the Folin–Ciocalteu reagent, following Singleton and Rossi [29].

A volume of 200 ml of the extract was mixed with 1 ml of Folin-Ciocalteu reagent diluted 10 times with water and 0.8 ml of a 7.5% sodium carbonate solution in a test tube. After stirring and 30 min later, the absorbance was measured at 765 nm by using a Jenway 6405 UV-vis spectrophotometer. Gallic acid was used as a standard for the calibration curve. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total flavonoid content

Total flavonoids were measured using a colorimetric assay adapted from Zhishen [30]. 1 ml aliquot of appropriately diluted sample (polyphenol extract corresponding to 1 g of dry plant material which was dissolved in 5 ml of methanol) or standard methanolic solution of catechin (at different concentrations) was added to 10 ml volumetric flask containing 4 ml double distilled H_2O. At zero time, 0.3 ml of sodium nitrite (5 %) was added to the flask. After 5 min, 0.3 ml of aluminium chloride (10 %) was added. At 6 min, 2 ml of NaOH (1M) was added to the mixture. Immediately, the reaction flask was diluted to volume (10 ml) with the addition of double distilled H_2O and thoroughly mixed. The absorbance was determined at 510 nm compared to control water. The sample was analyzed in triplicate and the average content was noted for each measure. The total flavonoid extracts were expressed as mg of catechin equivalents (CE) per g of dry weight of plant material (mg CE / g DW).

Total condensed tannins

phytochemical tests on water, alcoholic and etheric extracts showed no hydrolysable tannins and a strong presence of condensed tannins. Total condensed tannin content was measured using the modified vanillin assay described by Julkunen-Titto [31]. 1.5 ml of 4% methanol vanillin solution and 750 µl of concentrated HCL were added to 50 µl of sample. The mixture was kept for 20 min, and the absorbance was measured at 550 nm against methanol as a blank. The amount of total condensed tannins was expressed as mg catechin equivalent per gram of dry weight (mg CE/g DW) through the calibration curve with catechin. Triplicates measurements were taken for all samples.

Antioxidant activity evaluation

Ferric reducing antioxidant power assay (FRAP)

The reducing power was determined using the method of Oyaizu and Wang et al. [32, 33]. Five hundred microlitres of seed extract were added to1.25 ml of phosphate buffer (0.2 M, pH 6.6), 1.25 ml ferricyanide potassium and after incubation at 50 C for 20 min, 2.5 ml of trichloroacetic acid (10 % w/v) was then added to the mixture followed by centrifuging at 3000 rpm for 10 min. Consequently 1.25 ml trichloro-acetic acid (10%). The mixture obtained then, 1.25 ml of the mixture was picked and added to 1.25 ml of distilled water and 0.25 ml of ferric chloride (1%). The absorbance was measured at 700 nm. The results are expressed as equivalent mg Ascorbic acid (AAE) per 100 g of dry matter.

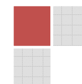
β-Carotene bleaching test

A modified method described by Koleva et al. [34] was employed. Β-Carotene (2 mg) was dissolved in 20 mL chloroform. Then, 4 mL of this solution were added to linoleic acid (40 mg) and Tween 40 (400 mg). Chloroform was evaporated under vacuum at 40 C and 100 mL of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. The emulsion (3 mL) was added to a tube containing 0.2 mL of different concentrations of the test emulsion was incubated in a water bath at 50 C for 120 min, when the absorbance was measured again. In the negative control, the extract was substituted with an equal volume of methanol. The antioxidant activity (%) was evaluated in terms of the bleaching of the β-carotene using the following formula: % Inhibition= (At – Ct / C0 – Ct) 100

where At and Ct are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min, and C0 is the absorbance values for the control measured at zero time during the incubation. The results are expressed as IC50 values (mg/mL), the concentration required to cause a 50% β-carotene bleaching inhibition.

Statistical analysis

Data are expressed as the means ± standard error of mean performed in triplicates. Statistical comparisons of the results



were determined by one-way ANOVA using Prism v6. software (GraphPad Software, 2014) followed by Tukey's and Dunnett's post test for multiple difference was considered to be statistically significant if the probability value comparison among the groups was less than 0.05 ($p < 0.05$).

Results and discussion

Physical and Chemical Parameters of Oil

The results show that oil content of citrus seed is 38.21 % which is in agreement with those reported for Nigerian *Citrus aurantium* seed (32.4%) [8], Tunisian citrus (Blood orange, Bitter orange and Bergamot) seeds (26.1–36.1%) [7] and Egyptian citrus (Citron, Orange, and Mandarin) seeds (38.9 to 42.6%) [39]. However the present oil yield was higher than Pakistan *Citrus aurantium* seeds (21.8 %) and was lower than those found in Tunisian sweet orange (51.8%) and lemon seeds (78.9%) [7].

The physico-chemical proprieties of extracted seed oil of *Citrus aurantium* are represented in Table 1. The present results compared well with those reported for Pakistan citrus seed oils acid value (0.50–2.18), density (0.920–0.941), refractive index (1.4639–1.4670), saponification value (180.90–198.85) [5]; and Egyptian citrus seed oils, refractive index (1.4650–1.4681) and density (0.913–0.933) [38]. However, values of saponification index (191.52) determined in the present analysis were higher than those reported of Nigerian *Citrus aurantium* seed (186±0.64) [8]. The refractive index (1.4671) determined in the present analysis agreed well with those reported for sunflower seed oils (1.467–1.469) [39]. These proprieties of *Citrus aurantium* seed oil may indicate its relative purity.

Table 1: Values of physicochemical index of seed oil of *Citrus aurantium*

Physicochemical index	Values
Density (20 C)	0,926 ± 0,024
Acid value	1,121 ± 0,115
Refractive index (22,3 C)	1,467 ± 0,060
Saponification value	191,52 ± 6,09
Ester index	190,41 ± 5,97

The data are displayed with mean ± standard deviation of twice replications.

The fatty acid composition of seed oil of *Citrus aurantium* is given in Table 2. Palmitic acid (26.85 %) was the major satured acid and linoleic acid (38.29%) was the major unsatured acid. There distributions were comparable with those reported for Nigerian *citrus aurantium* seed oil except for stearic acid (C18 :0) which is lower. The contents of oleic (30.87%) and linoleic (22.03%) acids reported for Pakistan *Citrus aurantium* seed oil [39] were different than those found in a present study. The content of -tocopherol is 194.25 µg/ml, this value is different than that observed in sunflower 0.6 mg/kg and cottonseed 3.3 mg/kg oils.

Table 2: Percent fatty acid and -Tocopherol composition of total lipids of *Citrus aurantium* seed.

fatty acid (%)	%
C16 :0	26.851
C18 :0	7.149
C18 :1	27.703
C18 :2	38.295
Satured	34
Unsatured	65.998
UFA/SFA	1,941
-Tocopherol (µg/ml)	194.25

Total phenolics content, total flavonoids and tannins of *Citrus aurantium* seed

The presence of phenolic compounds (flavonoids, tannins, others phenolic compounds) in *Citrus aurantium* seed Indicates that this seed may have the ability as an antioxidant agent.

Phenolic compounds are widely distributed in the plant kingdom. These compounds serve as important antioxidants because of their redox properties, which exhibit an important function in neutralizing free radicals. Hence, they prevent the oxidation of various biological molecules [40, 41].

Studies showed that the Citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids [42].

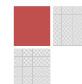
Principal results showed (Table 3) that *Citrus aurantium* seed extract exhibited amount of polyphenol content (2.126 mg GAE/g DW), followed by condensed tanins (0.3mg CAT/ g DW) and flavonoids fractions (Butanol fractions: 0.043mg CAT/g DW and Ethyl acetate fraction: 0.033 mg CAT/g DW).

Compared with amount phenolic compounds this values were higher than those reported by Moulehi et al. [43] (1.35mg GAE/g DW).

The amount of phenolic compounds observed in oil extracted from citrus seed is 0.219 mg GA / g DW (less than that showed in crude extract). Several oil seeds and their products have been investigated for phenolic compounds in search for safe sources of natural antioxidants [44].

The presence of phenolic compounds (flavonoids, tannins, others phenolic compounds) in *Citrus aurantium* seed Indicates that this seed may have the ability as an antioxidant agent.

Flavonoids have been reported to be responsible for anti-oxidant activity [45]. The antioxidant, anti-inflammatory, antifungal and healing properties of some plant extracts have been attributed to the presence of tannins [46].



From Tang et al. [47] the primary active biological constituents of sour orange are flavonoids, of which it has a high content.
In this study the flavonoids content ranged from 0.033 mg CAT / g DW (Ethyl acetate) and 0.043mg CAT / g DW (Butanol fractions) are lower than those reported in Tunisian *Citrus aurantium* seed varying from 1.5 to 1.8 GAE/g DW. However the tannin content (0.3mg CAT/ g DW) obtained in this study was quite similar to those found in Tunisian studies (0.37mg CAT/ g DW) [43]. The differences of the results obtained from this study compared to the previous findings could be attributed to the type of the cultivar, geographical origin of the fruits, degree of maturity, climatic conditions but also to the extraction protocols and analytic assays [48].

Table 3: Total Phenolic, Flavonoid and condensed Tannin Contents in *Citrus aurantium* seed

Extracts (polyphenol)	Total phenolic content (mg GA / g DW)	Total phenolic content in oil (mg GA / g DW)	Ethyl acetate fraction of flavonoid content (mg CAT / g DW)	Butanol fraction of flavonoid content (mg CAT/ g DW)	Total condensed tannins content (mg CAT/ g DW)
Citrus aurantium seed	2,126 ± 0,018	0.219±0,002	0,033 ± 0,004	0,043 ± 0,005	0,3 ± 0,03

The data are displayed with mean ± standard deviation of three replications.

Antioxidant activity evaluation

Several methods are used to evaluate the antioxidant activity by trapping different radicals [19]. In the present study, we applied two complementary test systems namely, β-carotene–linoleic acid and FRAP for evaluating the antioxidant capacities of different extracts of seed of Bigarade.

β-Carotene bleaching capacity

This technique consists in measuring, at 470 nm, discoloration of β-carotene resulting its oxidation by the decomposition products of linoleic acid, which produces hydroperoxides as free radicals during incubation at 50 C. Linoleic acid hydroperoxides attack the β-carotene molecule and, as a result, it undergoes rapid decolorization. The corresponding decrease in absorbance can be monitored spectrophoto-metrically. The addition of antioxidants pure [47] or in the form of plant extracts [34, 48] induces delay of the discoloration kinetics of β-carotene by hydroperoxides. The degradation rate of β-carotene linoleate depends on the antioxidant activity of the extracts.
Thus, we evaluated the antioxidant activity of *Citrus aurantium seed* extracts by the β-carotene linoleate bleaching method because β-carotene shows strong biological activity and is a physiologically important compound [40, 42].
The β-Carotene bleaching activity is usually expressed as percentage of inhibition but also by the antioxidant concentration required to 50% β-carotene bleaching inhibition. Basically, a higher percentage of inhibition is associated with a lower IC50 value.
The obtained results are summarized in table 4. These results are shown as relative activities against GA and BHA.
The results showed that all the extracts were capable of inhibiting the bleaching of β-carotene by scavenging linoleate-derived free radicals and were more effective in comparison with gallic acid. Most effective was tannins extract (0.20 ± 0.002 mg/ml) and ethyl acetat flavonoid extract (0.33 ± 0,013mg/ml) of *Citrus aurantium* seeds and were similar to high than gallic acid (0.43 ±0.001 mg/ml) but still lower than BHA (0,044 ± 0.002 mg/ml).
Lagha-Benamrouche and Madani [14] reported in their study on peels and leaves of varieties of Algerian orange that Bigarade presents the highest capacity to slow the rate of oxidation of linoleic acid and β carotene (77%) followed by Portugaise (71%).
The study conducted by Moulehi et al. [43] demonstrated that the antioxidant activities of seeds extracts of bitter orange were correlated with the polyphenols and linoleic acid inhibition. This positive correlation between the phenolic content and antioxidant capacity is reported [50] but recently it has been shown that the antioxidant activity of extracts is roughly connected to their phenolic composition and strongly depends upon their phenolic structures [50].

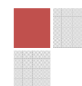
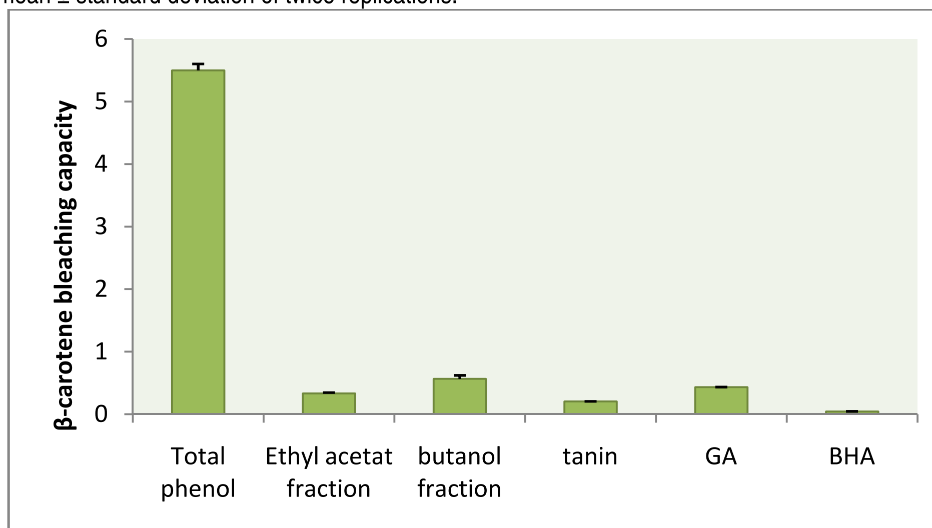


Table 4: β -Carotene bleaching capacity of *Citrus aurantium* seed extracts

	IC 50 (mg/ml)						
	Total polyphenol	Total phenol of oil	Ethyl acetate fraction of flavonoid	Butanol fraction of flavonoid	Tanins	GA	BHA
Citrus aurantium seed	5,49 ± 0,103	1,18±0,009	0,33 ± 0,013	0,56 ± 0,057	0,20 ± 0,002	0,43 ± 0,001	0,044± 0,002

The data are displayed with mean ± standard deviation of triplicate. GA : Gallic acid, BHA : butyleted hydroxyanisole

Figure n 1: β -Carotene bleaching capacity of *Citrus aurantium* seed extracts, GA: Gallic Acid, BHA: butylated hydroxyanisole. Results are expressed as mean ± standard deviation of twice replications.



Reducing antioxidant power assay (FRAP)

The ferric reducing antioxidant power assay measures the reducing ability of antioxidants against the oxidative effects of reactive oxygen species.

Electron donating antioxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce the ferric form (Fe^{3+}) to the ferrous form (Fe^{2+}). Prussian blue colored complex is formed by adding ferric chloride to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm [52, 53].

In this assay, yellow color of the test solution changes to green or blue color depending on the reducing power of antioxidant samples. Increasing absorbance indicates a high reducing power.

The analysis of the reducing power of different extracts (phenolic compounds, flavonoids and tannins) of *Citrus aurantium* seed, and standards (Ascorbic acid and BHA) using the potassium ferricyanide reduction method were described in Figure 1.

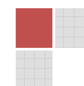
Among all samples, the ethyl acetate flavonoid extract present the most pronounced reducing power but significantly lower ($P < 0.05$) than that of standards.

This high reducing effect of the ethyl acetate extracted from seeds of *Citrus aurantium* might be attributed to their high quality, its confirmed by many recent studies which showing that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits such as the red grape, vegetables and medicinal plants [54, 55, 56].

However, butanol fraction flavonoid and tannin extracts were comparable but lower than that of the ethyl acetate extract .Mostly, the reducing properties are associated with the presence in the seeds of *Citrus aurantium*, compounds which exert their action by breaking the free radical chain by donating a hydrogen atom [57].

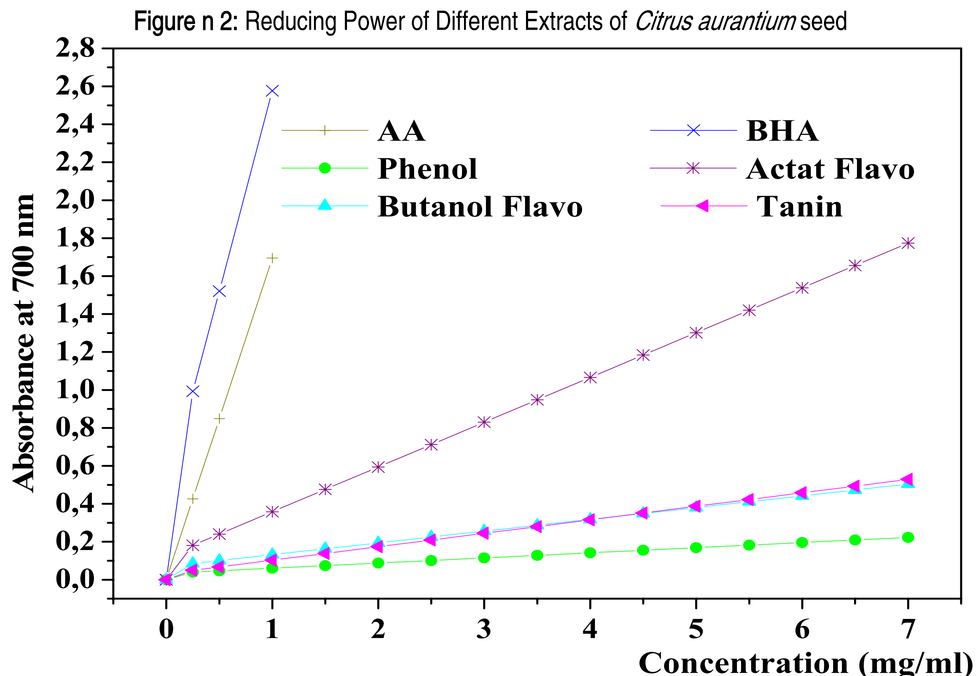
We have not observed the ferric reducing antioxidant in polyphenols extracted from oil of Citrus. It has been shown by Atolani et al. [58], orange seed oil had the lowest antioxidant potential among lime and grape seeds oils which they examined. [58]

They also conclude from their study that seed oils do not only possess the potential to act as natural antioxidant, but also



possess the ability to act as alternative and cheap source of oil as well as functional food for household purpose.

Figure n 2: Reducing Power of Different Extracts of *Citrus aurantium* seed



Conclusion

The results of the present investigation indicated that seeds of bigarade native to East of Algeria are rich of oil, which seems to be a good source of the insatured fatty acids and tocopherol. On the other hand, the evaluation of the antioxidant activity by β-Carotene bleaching test and ferric reducing antioxidant power (FRAP) showed that ethyl acetate followed by n-butanol fractions in *Citrus aurantium* seed have the highest values of antioxidant activity. Flavonoid extracts possess a higher antioxidant activity compared to other extracts.

These findings indicated that the seeds of *Citrus aurantium* provide components with potential for industrial and pharmacological applications as antioxidants. [43]

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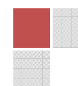

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