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Research Article

## Phenolic content and antioxidant activity of ethanolic extracts from *Citrus sinensis* L. and *Citrus reticulata* L. fruits

Assia Bentahar, Amel Bouaziz\*, Saliha Djidel and Seddik Khennouf

Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Natural and Life Sciences, Ferhat Abbas University Setif 1, Setif 19000, Algeria

### ABSTRACT

Citrus fruits, in particular the genus *Citrus*, are very rich in antioxidants which have beneficial effects on human health. The objective of this study was to evaluate the phenolic content and the antioxidant activity of the ethanolic extracts from orange (*Citrus sinensis* L.) and mandarin (*Citrus reticulata* L.) fruits. The quantitative estimation of the total phenolic content in the two extracts revealed their richness in these compounds. The results obtained showed that the polyphenols content were  $159.66 \pm 2.62$  mg GAE / g of dry extract and  $127.33 \pm 2.32$  mg GAE / g of dry extract for *C. sinensis* and *C. reticulata*, respectively. The quantification of flavonoids content showed that the ethanolic extracts of *C. sinensis* and *C. reticulata* contained  $0.85 \pm 0.01$  mg QE / g of dry extract and  $0.876 \pm 0.073$  mg QE / g of dry extract, respectively. Whereas, the tannins contents were  $46.32 \pm 1.02$  mg TAE / g of dry extract for *C. sinensis* and  $47.65 \pm 1.36$  mg TAE / g of dry extract for *C. reticulata*. *In vitro* antioxidant activity was evaluated using two tests: the reducing power and the hydroxyl radical scavenger test. The evaluation of the two extracts by the ferric iron reducing power test showed an  $EC_{50}$  value of  $0.882 \pm 0.037$  mg / ml for *C. sinensis* extract and an  $EC_{50}$  of  $1.085 \pm 0.068$  mg / ml for *C. reticulata* extract. In addition, *C. sinensis* and *C. reticulata* showed good hydroxyl radical scavenging effect with  $IC_{50}$  values of  $0.303 \pm 0.026$  mg / ml and  $0.572 \pm 0.100$  mg / ml for *C. sinensis* and *C. reticulata*, respectively. These results suggested that these fruit extracts could be good sources of phenolic compounds and ingredients with high antioxidant potential.

**Keywords:** *Citrus sinensis*, *Citrus reticulata*, Polyphenols, Flavonoids, Tannins, Antioxidant activity.

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\*Address for Correspondence:

Amel Bouaziz, Laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Natural and Life Sciences, Ferhat Abbas University Setif 1, Setif 19000, Algeria

### INTRODUCTION

Oxidative stress is an imbalance between oxidants and antioxidants. To protect the organism against oxidative stress, the body has a very complex antioxidant network composed of enzymes, iron and copper transporter proteins, vitamins, carotenoids, polyphenols, flavonoids, trace elements as selenium and other small molecules as glutathione. Most of these antioxidants are found in fruits and vegetables in their various organs either leaves, flowers or roots, which reinforces all their interest in the prevention of various pathologies<sup>1</sup>. Therefore, it is interesting to study medicinal plants and foods of plant origin that are daily and widely used in the field of biopharmaceutical research, due to their easy access, low cost and multiple therapeutic properties.

Citrus fruits are consumed around the world as an excellent source of vitamin C, which is a powerful natural antioxidant that strengthens the body's immune system and they have been used in traditional medicine as stomach tonic, astringent, carminative and antiscorbutic agents<sup>2</sup>. In addition, these fruits are used also to treat conditions such as constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, menstrual disorders, angina, hypertension, anxiety, depression and stress<sup>3</sup>. *Citrus* species exhibited numerous bioactivities as antioxidant<sup>4</sup>, anti-inflammatory<sup>5</sup>, antimicrobial<sup>6</sup>, anticancer, antiallergic<sup>4</sup> and neuroprotective activities<sup>7</sup>, as well as pulmonary fibrosis inhibitors<sup>8</sup>, anti-obesity and antihyperglycemic agents<sup>9</sup>. The aim of this work was to quantify the content of phenolic compounds and to study the *in vitro* antioxidant activity of the ethanolic extracts from the fruit pulp of *Citrus sinensis* and *Citrus reticulata*.

## MATERIALS AND METHODS

### Preparation of ethanolic extract

These citrus fruits were obtained from a market in Setif region, located in Northeastern Algeria. The fruits purchased were of good quality, without damage, washed in water, peeled, cut in half, seedless and quickly grinded. The ethanolic extract was obtained by maceration in water/ethanol mixture (20:80) for 5 days in the dark with agitation every 24 hours at. The resultant extract was filtered through Wattman paper and the solvent was removed by rotary evaporator under reduced pressure at 45°C<sup>10</sup>.

### Determination of total polyphenols content

The total phenolic content of the extracts was determined according to Folin-Ciocalteu method Li et al.<sup>11</sup>, with some modifications. 100 µl of each extract was mixed with 500 µl of Folin-Ciocalteu reagent (diluted 10 times) and incubated at room temperature for 4 min. Then, 400 µl of 7.5% sodium carbonate solution was added and further incubated for 1h 30 min at room temperature. The absorbance of all samples was measured at 760 nm and the results are expressed in milligrams of gallic acid equivalents per gram dried weight (mg GAE/g DW). All samples were analyzed in three replications.

### Determination of total flavonoids content

Measurement of flavonoid concentration in extracts was based on the method described by Bahorun et al.<sup>12</sup>. Each sample (1 ml) was added to 1 ml of aluminum chloride (AlCl<sub>3</sub>) solution (2%) and allowed to stand at room temperature for 10 min. The absorbance of the mixture was measured at 430 nm against the same mixture without the sample as a blank. Total flavonoid content was expressed as quercetin equivalent per gram dried weight (mg QE /g DW).

### Determination of tannins content

The capacity to precipitate hemoglobin was determined by using bovine fresh blood according to the method described by Gharzouli et al.<sup>13</sup>. Briefly, a volume of each fruit extract was mixed with an equal volume of hemolysed bovine blood (absorbance = 1.6). After 20 min, the mixture was centrifuged at 4000 rpm for 10 min, and the absorbance of the supernatant was measured at 756 nm. Results were expressed as mg equivalent tannic acid per gram dried weight (mg TAE/g DW).

### Evaluation of antioxidant activity

#### The hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method described by Smirnov et al.<sup>14</sup>. The reaction mixture contains 1ml of 1.5 Mm of FeSO<sub>4</sub>, 0.7ml of 6 Mm of hydrogen peroxide, 0.3ml of 20 mm of sodium salicylate and 1ml of the different concentrations of the extracts to be tested. After incubation for one hour at 37C °C, the absorbance is taken at a wavelength of 562 nm. The scavenger effect of the hydroxyl radical is calculated using the following equation:

$$I\% = 100 (A \text{ control} - A \text{ sample}) / A \text{ control}$$

Where A control is the absorbance of the blank solution (containing all reagents except the test compound), and A

sample is the absorbance in the presence of the test compound. Extract concentration providing 50 % inhibition (IC<sub>50</sub>) was calculated from the plot of inhibition percentage against extract concentration.

### Reducing power

The reducing power was measured using the method of Chung et al.<sup>15</sup>. An aliquot of each sample or BHT (0.1ml) was mixed with 0.1ml of Phosphate buffer (200mM, pH6.6) followed 0.1ml of 1% potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>]. After incubation in water bath at 50°C for 20 min, 0.25ml of 10% trichloroacetic acid was added into the mixture, and then followed by centrifugation at 3000 rpm for 10 min. Then the resultant supernatant (0.25 ml) was mixed with 0.25 ml of distilled water and 0.5 ml of 0.1 % ferric chloride (FeCl<sub>3</sub>), and the absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicated increased reducing power. The results are expressed in effective concentration at 50% (EC<sub>50</sub>) which reflected the concentration of antioxidant used to achieve an absorbance of 0.5.

### Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.01 for Windows). *In vitro* results were expressed as mean ± standard deviation (SD) and were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test. The P-values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

Extraction is an important step in the isolation of bioactive compounds from plants. The extraction process is based on the difference in solubility of the compounds of a mixture in a solvent<sup>16</sup>.

### Total polyphenols, flavonoids and tannins content

Plant polyphenols are secondary metabolites characterized by one or more hydroxyl groups binding to one or more aromatic rings. Several thousand polyphenolic molecules have been identified in higher plants, including edible ones. Plant polyphenols are divided into two major groups, flavonoids and non-flavonoids. Flavonoids can be divided into flavanols, flavonols, anthocyanidins, flavones, flavanones, and chalcones. Non-flavonoids include stilbenes, phenolic acids, saponins, and tannins<sup>17</sup>. Among the important biological properties exhibited by plant polyphenols, their antioxidant activity has raised a great interest<sup>17,18,19</sup>.

Table 1 represented the amount of total polyphenols, flavonoids and tannins contents in the ethanolic extracts from *C. sinensis* and *C. reticulata*. The results showed that the ethanolic extract of *C. sinensis* exhibited the highest total phenolic content (159.666 ± 2.625 mg GAE/g DW). While, the contents of flavonoids in ethanolic extracts from *C. sinensis* and *C. reticulata* are close with values of 0.85 ± 0.012 mg QE/g DW and 0.876 ± 0.073 mg QE/g , respectively. Also, the results showed the richness of orange and mandarin extracts in tannins with converging values (46.32 ± 1.02 mg TAE/g and 47.65 ± 1.36 mg TAE/g, respectively).

**Table 1:** Total polyphenols, flavonoids and tannins contents in ethanolic extracts from *C. sinensis* and *C. reticulata* fruits.

Extract	Polyphenols <sup>(a)</sup>	Flavonoids <sup>(b)</sup>	Tannins <sup>(c)</sup>
<i>C. sinensis</i>	159.666 ± 2.625	0.85 ± 0.012	46.32 ± 1.02
<i>C. reticulata</i>	127.333 ± 2.322	0.876 ± 0.073	47.65 ± 1.36

(a)mg Gallic acid Equivalent /g of dry extract ; (b) mg Quercetin Equivalent / g of dry extract ; (c) mg Tannic acid Equivalent /g of dry extract ; Results are expressed as means ± SD (n = 3).

Abd ghafar et al. <sup>20</sup> reported that orange ethanolic extract had a total phenolic content of 135.3 ± 0 mg GAE / g DW. While, Canan et al. <sup>21</sup> showed that the total polyphenol content of orange methanolic extract was 270.56 ± 0.67 mg GAE / g DW and mandarin methanolic extract contained 302.38 ± 0.91 mg GAE / g DW. Recently, Zhang et al. <sup>22</sup> have reported that the quantitative estimation of total polyphenols and flavonoids in the methanolic extract of mandarin were 8.25 ± 0.21 mg GAE / g DW and 6.38 ± 2.21 mg QE / g DW, respectively. Ultimately, it is difficult to compare our results with those in the bibliography, because of several factors which can influence the qualitative and quantitative distribution of phenolic compounds in the extracts. among these factors: the chemical nature of the compounds, the extraction method and the solvents used, the solubility of the phenolic components <sup>23</sup>, the origin of the plants and their cultivation and the harvest season <sup>24,25</sup>, climatic and environmental conditions (high temperature, high exposure to the sun, drought and salinity) <sup>26</sup>, the different diseases that can attack them, the use of a different standard and a different calibration curve are also factors that must be taken into consideration <sup>27</sup>.

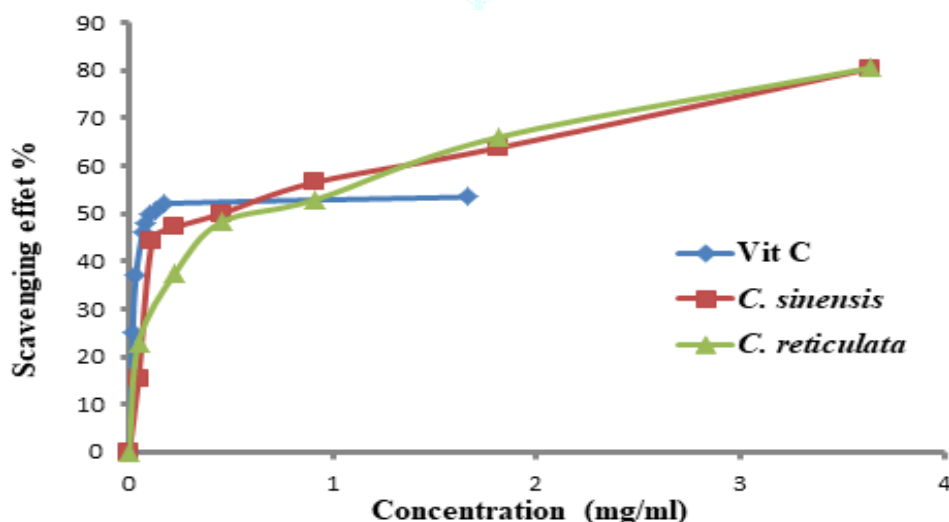
### Evaluation of antioxidant activity

Polyphenolic compounds clearly improve the status of different oxidative stress biomarkers. The biological mechanisms of these possible effects have been attributed to their antioxidant properties through several possible mechanisms, such as their ability to scavenge free radicals, break radical chain reactions, directly reducing peroxides, and stimulating the antioxidative defense enzyme activities <sup>28</sup>.

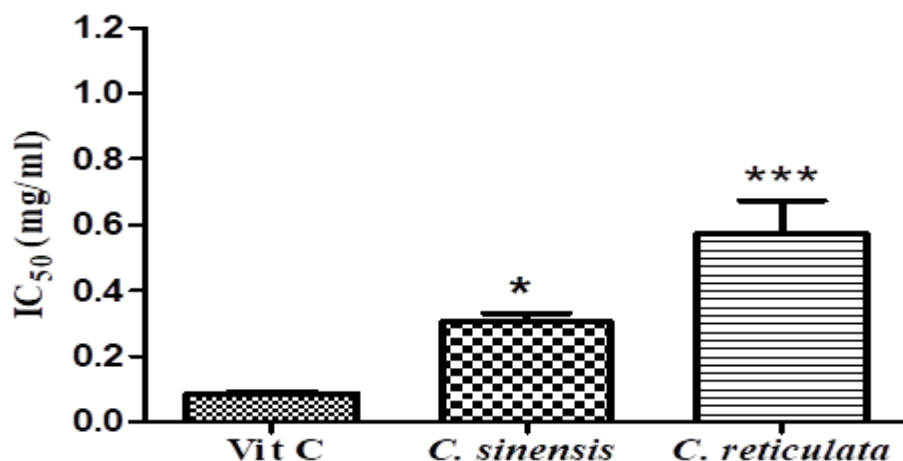
### Hydroxyl radical scavenging activity

This test consists of scavenging the hydroxyl radical formed through the Fenton reaction to the reaction medium with sodium salicylate and forming a pink complex observed at an absorbance of 562 nm.

In this study, a significant decrease in the amount of hydroxyl radicals was observed due to the scavenging ability of standard and fruit ethanolic extracts (Figure 1). Figure 2 revealed that the ethanolic extract of *C. sinensis* exhibited a strongest antioxidant activity (IC<sub>50</sub> = 0.303 ± 0.026 mg / ml, p < 0.05), which is comparable to the standard vit C (IC<sub>50</sub> = ± mg / ml). The ethanolic extract of *C. reticulata*, also showed good hydroxyl radical scavenging activity with an IC<sub>50</sub> = 0.572 ± 0.100 mg / ml (P < 0.001).



**Figure 1:** Hydroxyl radical scavenging activity of vitamin C and fruit ethanolic extracts.



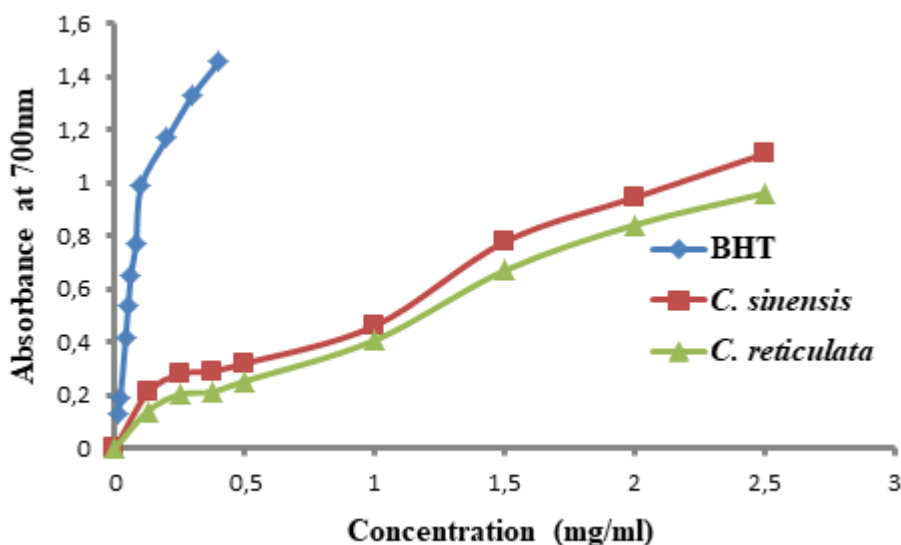
**Figure 2:** A comparison between ethanolic extracts of *C. sinensis* and *C. reticulata* in hydroxyl free radical scavenging activity. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n = 3). (\*p < 0.05, \*\*\* p < 0.001) compared to vit C as standard.

The obtained results may be due to the presence of polyphenols and flavonoids which have the principal contribution to the antioxidant capacity of fruits extracts. In fact, literature showed that good correlation was found between antioxidant activity and the content of polyphenols and flavonoids contents<sup>19, 29</sup>. Albano and Miguel<sup>30</sup> reported that flavonoids and phenolic acids are more effective in scavenging free radicals after extraction with moderate or hydrophilic solvents. In addition, tannins are excellent scavengers of free radicals, such as iron and copper in free form because of their phenolic functions, which have a strong nucleophilic character<sup>31,32</sup>.

#### Reducing power capacity

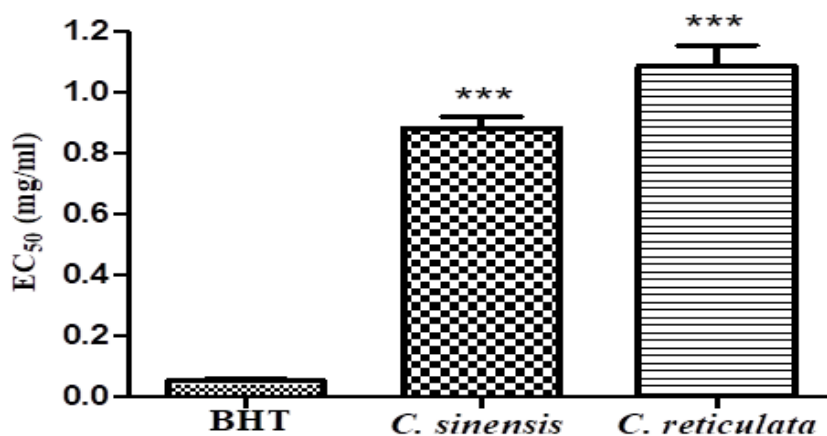
The reducing capacity of the extracts, another significant indicator of antioxidant activity. It is attributed to reductants, which have hydrogen-donating ability, resulting in potent antioxidant activities in test samples. In addition, antioxidant activities have been reported to be directly correlated with reducing power abilities in some plant-based compounds<sup>28</sup>.

As shown in Figure 3, the reducing capacity was proportional to the concentration of ethanolic extracts of the two fruits and BHT as standard. As shown also in Figure 4, there was significant difference (p < 0.001) among extracts in the reducing power with EC<sub>50</sub> values of  $0.882 \pm 0.037$  mg/ml and EC<sub>50</sub> of  $1.085 \pm 0.068$  mg/ml for *C. sinensis* and *C. reticulata* extracts, respectively compared to BHT as positive control (EC<sub>50</sub> =  $0.051 \pm 0.006$  mg/ml).



**Figure 3:** Reducing power capacity of standard and fruit ethanolic extracts.





**Figure 4:** A comparison between ethanolic extracts of *C. sinensis* and *C. reticulata* in reducing power assay. Data were presented as EC<sub>50</sub> means  $\pm$  SD (n = 3). (\*\*\*) p < 0.001 compared to BHT as standard.

Wang et al.<sup>33</sup> showed that antioxidant activities have been reported to be directly correlated with reducing power abilities in some plant-based compounds. In addition, the increase in power reduction capacity may be due to the formation of reducing agents, which react with free radicals to stabilize and terminate free radical chain reactions during fermentation<sup>34</sup>.

## CONCLUSION

This study was the first report about the antioxidant activities of two species of *Citrus* which are commonly available in Algeria. The results of this study showed that the ethanolic extracts of *C. sinensis* and *C. reticulata* possessed high phenolic and tannins content and exhibited good antioxidant activity by the hydroxyl radical scavenging activity and reducing power capacity methods. The use of *C. sinensis* and *C. reticulata* fruits as a natural antioxidant source appears to be an alternative to synthetic antioxidants. Further investigations are needed to determine antioxidant activity of *Citrus* species by *in vivo* methods and the exact mechanism of action for their pharmacological properties.

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