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STUDY OF THE ANTIOXIDANT PROPERTIES OF THE WATER EXTRACT IN SYRIAN CITRUS LIMON AND CITRUS AURANTIUM LEAVES

Thawab Alghabra[1], Manal Daghestani[2], Amina Ibrahim[3])

[1] PhD., Student, [2]Assistant Professor, Department of Chemistry, Faculty of Sciences, Damascus University, Syria,

[3]Doctor, Department of Chemistry, Faculty of Sciences, Damascus University.

Abstract:

Citrus are well-known fruits which possess enormous medicinal properties. This research aimed to investigate the most effective compounds (phenolics and flavonoids), in the water extract's leaves of two essential Syrian Citrus "Citrus limon and Citrus aurantium" using conventional spectrophotometric technique. The antioxidant activity of Citrus limon and Citrus aurantium was analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3- ethylbenzothiazoline-6-sulphonic acid] (ABTS) assays, the IC_{50} values of DPPH and ABTS free radicals were measured. Vitamin C was used in this research as the positive antioxidant control. The results indicated that total phenolics (TPC) and total flavonoids content (TFC) for water extract's leaves of Citrus aurantium (TPC=95.70±0.80 mg/g, and TFC=64.20±0.70 mg/g) were higher than those of Citrus limon (TPC=128.11±0.50 mg/g, and TFC=103.60±0.50 mg/g). Furthermore, the water extract's leaves of Citrus aurantium exhibited potent scavenger of free radicals, with IC_{50} values of 22.5±0.700 µg/ml and 27.5±0.050 µg/ml for DPPH tests, respectively. Whilst the water extract's leaves of Citrus limon and Citrus aurantium's radical potential, with IC_{50} values of 2.4±0.40 µg/ml and 3.33±0.40 µg/ml for ABTS tests, respectively. Based on acquired results, both water extract's leaves of Syrian Citrus could be recommended as a promising source of bioactive compounds valuable for avoiding threating diseases connected to oxidative stress such as cancer, aging, atherosclerosis, cardiovascular and neurodegenerative diseases.

Keywords: antioxidant, Total phenols, Total flavonoids, DPPH., ABTS+., Citrus limon leaves, Citrus aurantium leaves

1.Introduction:

Ancient civilizations used medicinal plants as the main therapeutic agents against numerous diseases since they provided essential source for biologically active compounds. At present these medicinal plants are still used by almost 80% of the world inhabitants for their healing properties [1,2]. The medicinal properties of plants are linked to their phytochemical contents such as phenolic acids, flavonoids, tannins, and alkaloids [3]. Phenolics and flavonoids compounds are plant's secondary metabolites [4], which have been correlated with antioxidant activities [1]. Antioxidant's molecules are capable of delaying or inhibiting other molecule's oxidation caused by reactive oxygen spices (ROS) [5] including "free radicals". These free radicals can attack the body's healthy cells, which cause them to lose their structure and function, this oxidative damage contributes to several health diseases such as aging, cancer, brain dysfunction, cardiovascular disease, and immune system decline [6]. Consequently, plant metabolites "antioxidants" [7] are extremely important to maintain optimum cellular and systemic health, along with well-being [6]. Globally, citrus fruits belonging to the family Rutaceae [8], are one of the most essential crops [9], that possess natural sources of antioxidants [10]. Citrous is famous for having significant amounts of vitamin C, flavonoids, and phenolic compounds, thus providing nourishing benefits [9,10], and countless medicinal properties against tumor, coronary diseases, blood clotting, and asthma [8]. Citrus limon and Citrus aurantium that originated in Syria and generally consumed as fresh fruits and juices, are important crops [11,12], which have a great role in the nutraceutical values [4]. Several studies reported the massive antioxidant activities [7] and the beneficial effects of Citrus limon and Citrus aurantium fruits on human health [3,7], due to owning high quantity of phenolic compounds (Fig.1) [13]. Citrus limon is a tree with green leaves and yellow fruits, its pharmacological effects include anticancer, antioxidant, antimicrobial, antiparasitic, and anti-inflammatory properties [13,14]. While Citrus aurantium is a small tree with bitter orange and scented white flowers [12], it possesses many therapeutic activities which is effective in the treatment of lung and prostate cancers, gastrointestinal disorders, obesity, and anxiety [15]. Recently, a great interest has raised to obtain natural antioxidants as a replacement for the synthetic antioxidants, as many literatures indicated significant health risks anticipated from long-term consumption of synthetic antioxidants, like gastrointestinal tract complications, skin allergies, in addition they are believed to cause cancer [16]. Even though the non-edible parts (peels, leaves and seeds) [8] of citrus fruits are rich in bioactive compounds [3], their antioxidant properties were not

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widely investigated [10]. Hence, it was the purpose of this study to appraise the phenolic constituents and their natural antioxidant capacity of the water leaves extract of two Syrian citrus species "Citrus limon and Citrus aurantium".

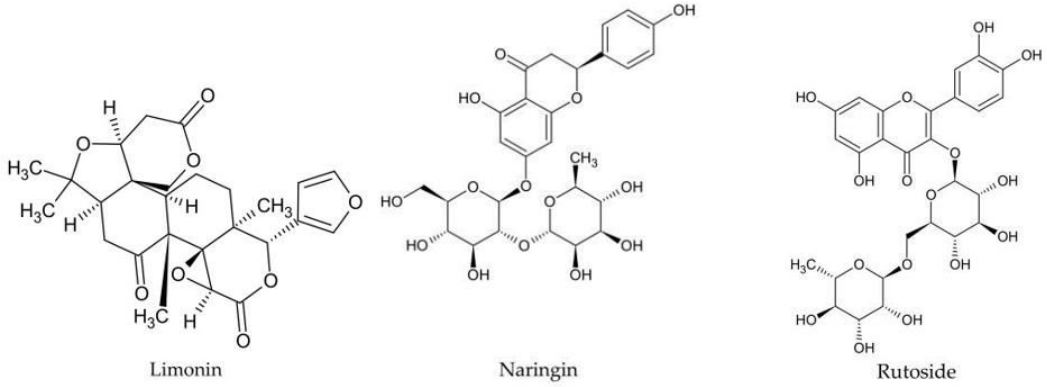

Figure 1. Chemical structures of major phenolic compounds found in Citrus leaves.

2. Materials and Methods

2.1. Plant Materials

Citrus leaves were collected from several trees within houses in old neighborhoods of Damascus (Syria), at the beginning of March 2019. The leaves of the Citrus limon were classified from the rational type with green broad leaves, whereas the leaves of the Citrus aurantium were classified from the wild Indian hybrid type, depending on the characteristics of the plant Contained in the Syrian Flora and Atlas of Medicinal and Aromatic Plants in the Arab World (Al-Hakim and colleagues, 2012).

2.2. Chemicals and Reagents

Ethanol 99.8% (GC), anhydrous sodium carbonate (99.5%), Folin– Ciocalteu reagent, aqueous Gallic acid (97.5-102.5%), aluminum chloride solution, potassium acetate solution (1M), quercetin 98%(HPLC-grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Reagent grade), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) solution 99.0% (HPLC-grade), and ascorbic acid. All other solutions were of analytical grade and were obtained/ purchased from sigma

2.3. Instruments

Circular mini mixer (Vortex) MS1 Minishaker (KAI), Ultrasound water bath Model 460 / H (Elma) Transsonic, Hygrometer (Sartorius / MA35), and UV-VIS Spectrophotometer (JASCO).

2.4. Extraction Procedures

One gram of dry plant leaves was soaked in 20 ml of distilled water, these water solution mixtures were selected for its capability to achieve a good quality of polyphenol's extraction [17], besides its toxicity is less than other solutions [18]. The mixtures were well stirred with a circular mixer (for homogenization) for one minute, then put in the ultrasound machine at 40 °C for 30 minutes. Ultrasonic extraction technique was applied to speed up the release of bioactive materials from cell walls, as well to facilitate their transference, thus enhancing the extraction [19]. After that, the extracts were filtered with filters (0.45 μm) and kept in the refrigerator at 7 °C for 24 hours. Each extraction process was carried out three times.

2.5. Qualitative detection of the active compounds of the water extracts of Citrus limon and Citrus aurantium leaves

Traditional methods for qualitative detection of the most important natural bioactive compounds found in citrus leaves extracts were conducted according to what was previously reported in [20]. In this study tannins, flavonoids, saponins and alkaloids were identified by the following analytical procedures Trible Iron Chloride method, Shinoda reaction method, Saponin foaming test, and Meyer's reagent test, respectively.

2.6. Determination of total phenol content (TPC)

The Folin-Ciocalteu method was applied to evaluate the total phenol content (TPC) as previously mentioned in [21]. 1 mL of the 70% ethanol diluted sample was mixed with 4.8 mL di-distilled water, 4 mL anhydrous sodium carbonate (2% w/v), and 200 μl of Folin-Ciocalto reagent, then the mixture was left in a dark place at room temperature for an hour. The absorbance was recorded at 760 nm using a UV-VIS spectrophotometer (JASCO) and compared to the control sample (1 ml of ethanol with the same sequence of additives without the sample) [22,23]. A linear standard curve was constructed from

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several concentrations (0-150 mg/ml) of gallic acid in 70% ethanol. The total phenol content was expressed as gallic acid, and the results were expressed as mg of gallic acid equivalents (Ga_{Es}) per g of dry powder (DP). Total phenolic content was determined according to the following equation:

$$y = 0.004x - 0.0012$$

y is the absorbance at 760 nm, and x is the total phenolic content in extracts. [27]

2.7. Determination of total flavonoid content (TFC)

The colorimetric method based on the formation of a yellow flavonoid–aluminum chloride complex [1], was performed to estimate the total flavonoid content (TFC) spectrophotometrically as reported by [Shaghaghi et al., 2009 OR 2OO8 (23)]. 1 ml of 70% ethanol diluted sample was added to 3 ml of 99.5% ethanol, 200 µl of aluminum chloride solution (10% w/v), and 200 µl of potassium acetate solution (1 M), then followed by the addition of 5.6 ml of deionized water. The solution was well mixed and left in a dark place at room temperature for 40 minutes. The absorbance was recorded at 440 nm and compared to the control sample (1 ml ethanol with the same sequence of previous additions without the sample) […Shaghaghi et al., 2009 OR 2OO8 (23) ???.] A calibration curve was prepared from different concentrations (0-100 mg/l) of quercetin in 70% ethanol. The results were expressed as mg of quercetin equivalents (Q_{Es}) per g of dry powder (DP). Total flavonoid content was determined according to the following equation:

$$y = 0.008x - 0.00001$$

y is the absorbance at 440 nm, and x is the total flavonoid content in extracts. /// [27]

2.8. Antioxidant activities

2.8.1. DPPH radical-scavenging activity assay

The principle of this test is based on the reduction of violet-colored of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical [3], when reacting with any antioxidant which can donate a hydrogen atom, to form a yellow-colored diphenylpicrylhydrazine [24]. The free radical scavenging activity was determined spectrophotometrically according to [25]. 300 µg of each extracts of citrus leaves at various concentrations within the range (1- 0.2 mg/ml) was added to a test tube, then 3 ml of a solution of 45 µg/ml $DPPH^{\bullet}$ in ethanol was added to the tube. After shaken strongly, all tubes were kept in a dark place at room temperature for 30 minutes. The absorbance was determined at 515 nm wavelength and compared to the control sample. Various concentration range of standard vitamin C (0.1-0.02 mg/ml) was used with all reagents except the sample at the same conditions. $DPPH^{\bullet}$ scavenging activity (%) was calculated from absorbance values according to the following equation:

$$I_{DPPH^{\bullet}}\% = [(A_b - A_a)/A_b] \times 100$$

$I_{DPPH^{\bullet}}\%$ is the inhibition percentage of DPPH radical scavenging capacity, A_a is the absorbance of the sample, and A_b is absorbance of the control sample. Antioxidant activity was expressed by IC_{50} value, which represents the sample's concentration needed to cause a 50% reduction in initial $DPPH^{\bullet}$ concentration [5,26]. The IC_{50} (mg/mL) values were calculated by linear regression analysis, where the percentage of $DPPH^{\bullet}$ scavenging activity was plotted against the sample's concentration [27].

2.8.2. ABTS radical scavenging assay

According to this assay, the 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) radical cation of blue green color, is generated when ABTS is oxidized with potassium persulfate. The radical scavenging capacity of antioxidants is determined when the intense blue green $ABTS^{\bullet+}$ is converted back to the colorless ABTS [3,24]. The free radical scavenging activity was measured according to the technique stated in literature [28]. The $ABTS^{\bullet+}$ solution was prepared by mixing two equal volumes of 7 mM ABTS solution and 2.45 mM of sodium persulphate (Na_2S2O_8) solution. The mixture was left in the refrigerator in the dark for a full night before using it. The solution was diluted with absolute ethanol to get an absorbance of (0.7 ± 0.02) at 734 nm. 200µl of extracts of various concentrations ranging (0.2-1 mg/ml) were placed in 200 µl test tubes, then 3 ml of $ABTS^{\bullet+}$solution in previously ethanol 70% was added to each tube. After stirring, the tubes were placed in a dark place at room temperature for 10 minutes. The absorbance was measured at 734 nm and compared to the control sample. Titrated series of vitamin C (0.02-0.08 mg/ml) was used with all reagents except the sample at the same conditions. $ABTS^{\cdot+}$ scavenging activity (%) was calculated from absorbance values according to the following equation:

$$I_{ABTS^{\cdot+}}\% = [(A_b - A_a)/A_b] \times 100$$

$I_{ABTS^{\cdot+}}\%$ is the inhibition percentage of ABTS radical scavenging capacity, A_a is the absorbance of the sample, and A_b is absorbance of the control sample. Like DPPH assay, the antioxidant activity was expressed by IC_{50} value, which represents the sample's concentration needed to cause a 50% reduction in initial $ABTS^{\bullet+}$concentration [5,26]. The IC_{50} (mg/mL) values were calculated by linear regression

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analysis, where the percentage of $ABTS^{\bullet+}$ scavenging activity was plotted against the sample's concentration [27].

2.8.2. Statistical Analysis

The results were analyzed statistically using the 20.0IBM-SPSS program. All experiments were carried out 3 times with 95% confidence level (α = 0.05), the data were expressed as mean ± standard deviation (SD), and IC_{50} values were calculated from the linear regression analysis.

3. Results and Discussion

3.1. Determination of moisture in the plant

The percentage of moisture M% was measured in both citrus leaves, with the following values: M% = (17.28 ±1.00) and (23.00 ± 2.00) for Citrus limon and Citrus aurantium leaves, respectively. As Citrus aurantium leaves have a larger water content, this indicates a greater capability to reserve water than Citrus limon leaves [27].

Additionally, the dry weight of Citrus leaves, were also calculated in the water extract, the results showed that the water extract of Citrus limon gave a higher yield than the ethanol extract of Citrus aurantium. These values are matching /higher /smaller than the values mentioned in [7].

3.2. Qualitative detection of the active compounds of the water extracts of Citrus limon and Citrus aurantium leaves:

Results from the qualitative analysis, which was conducted according to the methods mentioned earlier, to detect the bioactive compounds found in the water extracts of both Citrus limon and Citrus aurantium leaves, exhibited the presence of tannins, flavonoids, and saponins, while alkaloids were absence in both extracts, as shown in Table (1). Although, the results of tannins, flavonoids, and saponins, were positive and matching those in previous literatures. Yet the test for alkaloids was positive for Nigerian Citrus limon leaves extract according to [29], and positive for Indian Citrus aurantium leaves extract as reported in [30].

Table 1. Results of the qualitative detection of active compounds in the water extracts of Citrus limon and Citrus aurantium leaves.

Test	Citrus aurantium	Citrus limon
Tannins	+	+
Flavonoids	+	+
Saponins	+	+
Alkaloids	–	–

3.3. Determination of total phenolic and total flavonoid contents

Table (2) exhibited the total polyphenol content in the water extracts of Citrus limon and Citrus aurantium leaves, where ethanol was used as an extract, since it is less toxic than methanol [31]. TP and TF contents of Citrus limon leaves extract were significantly lower than those of Citrus aurantium. It was notable that TF value of Citrus limon (37.68±0.50 mgQ_{Es}/g) displayed almost one-third of that of Citrus aurantium (98.96±0.70 mgQ_{Es}/g). Additionally, these results were higher than the results recorded in an Indian study [7] and stated that TP contents of Citrus limon and Citrus aurantium leaves extracts were in the range (7.39 to 33.05 µg PCE/mg), and TF contents were within the range (0.51 to 21.62 µg QCE/mg). Another study performed in Iraq [32] has reported that the water extracts of Citrus aurantium had as well higher amounts of TP and TF (242.8 GAE/g dw, and 123.5 mg/100 g dw, respectively), than those of Citrus limon (TP were approximately 150 GAE/g dw, and TF within the range (123.5- 85.3 mg/100g dw), in addition its values were more comparing with this study. Thus, according to present and previous studies Citrus aurantium's leave extracts have larger quantities of TP and TF contents compared to those of Citrus limon.

Table 2. TP and TF values for water extracts of Citrus limon and Citrus aurantium leaves.

Water extract	TP $mgGa_{Es}/g$ DP	TF mgQ_{Es}/g DP
Citrus limon	128.11 ± 0.50^{a}	103.60 ± 0.50^{c}
Citrus aurantium	95.70 ± 0.70^{b}	64.20 ± 0.70^{d}

Ga_{Es}: Gallic acid Equivalents, Q_{Es}: Quercetin Equivalents, and DP: Dry Plant leaf powder. Different letters (a, b, c, and d) in the same column indicate the presence of significant differences

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in the content of TP and TF between the leaves of two types of citrus species, according to the independent-sample T test with the level of significant differences 0.05 $\alpha = 0.05$**.**

3.4. Antioxidant activities

It is noted that the antioxidant activity strongly related to the phenolic compound content of the plant extracts [33]. DPPH and ASTB assays based on the ability of an antioxidant to reduce an oxidant, thus color changes upon reduction, and the amount of color change is linked to the sample's antioxidant power. Therefore, these tests were performed to examine the antioxidant abilities to deactivate the free radicals in the water extracts of Syrian Citrus limon and Citrus aurantium leaves. According to these tests a smaller IC_{50} value suggests a higher antioxidant activity [5].

3.4.1. DPPH radical-scavenging activity assay

It was observed that the water extract of Citrus aurantium leaves has stronger effect in DPPH radical scavenging than that of Citrus limon leaves. The IC_{50} value (20.3±0.440 mg/ml) of Citrus limon extract was almost twice the IC_{50} value (11.9±0.700 mg/ml) of Citrus aurantium extract. Still both extracts are believed to possess strong antioxidant activities according to previous research [4], since IC_{50} values of both extracts are within the range 10-50 mg/mL. On the other hand, the value of IC_{50} of Citrus aurantium extract was remarkably higher than that of Vitamin C (the standard) Table (3), thus exhibiting a lower scavenging effect comparing with Vitamin C. This agrees with previous literatures which indicated that in determining the antioxidant power, the phenolic compounds in citrus fruits contribute less than vitamin C [10]. According to the Indian study that mentioned earlier, the IC_{50} value of Citrus aurantium extract was (142.25±0.86 µg/ml), while the IC_{50} value of Citrus limon extract was not detectable [7]. This could be due to the low amount of TF contents of the extracts resulting in weaker antioxidant activities [33] compared with this work. Moreover, it is not usually possible to evaluate antioxidant activities using the DPPH or ABTS assays with those registered in the literature, since the IC_{50} data depend on the reaction time, so relevant comparisons can only be made under the same protocols [24]. Interestingly, The IC_{50} values of the mwater extracts of Citrus limon and Citrus aurantium leaves from a study carried out in Algeria [3], showed that Citrus aurantium leaves extract has also more scavenging effect (68.44±3.71µg/mL) than that of Citrus limon (78.23±1.57 µg/mL). Accordingly, Citrus aurantium leaves revealed a high antioxidant ability to inhibit $DPPH^{\bullet}$ in the water and mwater extract. In water extract Citrus limon leaves has nearly half the scavenging power of Citrus aurantium leaves.

Table 3. IC_{50} value of DPPH radical scavenging activity

Sample	IC_{50} µg/ml
Vit C	5.2 ± 0.10^{a}
Citrus limon	27.4 ± 0.50^{b}
Citrus aurantium	22.5 ± 0.70^{c}

Different letters (a, b, and c) indicate significant differences in the IC_{50} values for each of the water extract of the two plants, and vitamin C as the standard in the DPPH test, according to SPSS program.

3.4.1. ABTS radical-scavenging activity assay

Similarly, to the DPPH test's results the IC_{50} value of ABTS scavenging activity of Citrus aurantium leaves extract (24.5±0.050 µg/ml) was lower than that of the IC_{50} value of Citrus limon leaves extract (45.3 ± 0.040mg/ml) which indicated a higher inhibiting power, yet when compared with IC_{50} value of Vitamin C, it demonstrates a remarkable lower scavenging activity. Table (4). Whereas according to the IC_{50} values obtained from the mwater extracts of Citrus limon and Citrus aurantium leaves in the Algerian study [3], surprisingly Citrus limon leaves extract (169.22±0.91 µM TE/g) was more powerful in inhibiting $ABTS^{\bullet+}$ than Citrus aurantium leaves extract (353.48±3.81µM TE/g). Hence, Citrus aurantium leaves is more successful in reducing $ABTS^{\bullet+}$ in the water extracts than Citrus limon extract. On the contrary Citrus limon extract has a better scavenging effect in the mwater leaves extract.

Table 4. IC_{50} value of ABTS radical scavenging activity

Sample	IC_{50} µg/ml
Vit C	4.0 ± 0.100^{a}
Citrus limon	2.54 ± 0.40^{b}
Citrus aurantium	3.33 ± 0.40^{c}

Different letters (a, b, and c) indicate significant differences in the IC_{50} values for each of the water extract of the two plants and vitamin C as the standard in the ABTS test, according to SPSS program.

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Based on the obtained IC_{50} values of DPPH and ABTS assays, the water extract of Citrus aurantium leaves provide a higher scavenging power the water extract of Citrus aurantium leaves. As a result, Citrus aurantium leaves own a stronger antioxidant activity than Citrus limon leaves.

4. Conclusion

In conclusion this work examined and compared the antioxidant capacity of the water extracts of the leaves of two major citrus fruits in Syria "Citrus limon and Citrus aurantium". From the qualitative analysis it was found that tannins, flavonoids, and saponins are the most active compound presents in both leaves extract. While the quantitative analysis of total polyphenol contents showed that Citrus aurantium leaves extract has greater amount of total phenolic and flavonoid contents compared to Citrus limon leaves extract. Moreover**,** the results obtained from DPPH and ABTS assays confirmed that the water leaves extract of Citrus aurantium has more power to inhibit both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ than Citrus limon extract, since Citrus aurantium is richer with bioactive compounds. Consequently, Syrian Citrus limon and Citrus aurantium leaves could be considered as good supplies for natural bioactive compounds with appropriate antioxidant activities and anti cancer drugs .

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