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# INVESTIGATION OF THE PHYTOCHEMICAL COMPOSITION AND ANTIOXIDANT PROPERTIES OF LEMON FRUITS

***©Rahimova S.**, ORCID: 0009-0003-5867-5254, Nakhchivan State University, Nakhchivan, Azerbaijan, rahimovasure@ndu.edu.az*
***©Alizade N.**, ORCID: 0009-0002-2380-4063, Nakhchivan State University, Nakhchivan, Azerbaijan, alizadenuran23@gmail.com*

# ИССЛЕДОВАНИЕ ФИТОХИМИЧЕСКОГО СОСТАВА И АНТИОКСИДАНТНЫХ СВОЙСТВ ПЛОДОВ ЛИМОНА

***©Рахимова С.**, ORCID: 0009-0003-5867-5254 Нахчыванский государственный университет, г. Нахчыван, Азербайджан, rahimovasure@ndu.edu.az*
***©Ализаде Н.**, ORCID: 0009-0002-2380-4063, Нахчыванский государственный университет, г. Нахчыван, Азербайджан, alizadenuran23@gmail.com*

*Abstract.* The phytochemical composition of lemon (*Citrus limon*) fruits was investigated, focusing on phenolic compounds, flavonoids, and antioxidant activity. The extracts were analyzed to determine their total phenolic and flavonoid contents using spectrophotometric methods. Gallic acid and catechol were used as standards for the quantification of total phenolics and flavonoids, respectively. The antioxidant activity of the extracts was evaluated using different in vitro assays, including the DPPH radical-scavenging assay and reducing power. The results demonstrated that the lemon fruit extracts contained considerable amounts of phenolic and flavonoid compounds and exhibited significant antioxidant activity. These findings suggest that Citrus limon fruits are a valuable source of bioactive phytochemicals and may have potential applications as natural sources of antioxidants.

*Аннотация.* Было проведено исследование фитохимического состава плодов лимона (*Citrus limon*) на содержание фенольных соединений, флавоноидов и антиоксидантную активность. Экстракты анализировались для определения общего содержания фенольных соединений и флавоноидов с использованием спектрофотометрических методов. В качестве стандартных соединений для количественного определения общего содержания фенольных соединений и флавоноидов использовались галловая кислота и катехол соответственно. Антиоксидантная активность экстрактов оценивалась с помощью различных тестов in vitro, включая тест на поглощение радикала DPPH и тест на восстановительную способность. Результаты показали, что экстракты плодов лимона содержат значительное количество фенольных и флавоноидных соединений и обладают высокой антиоксидантной активностью. Эти данные свидетельствуют о том, что плоды Citrus limon являются ценным источником биологически активных фитохимических веществ и могут иметь потенциальное применение в качестве природных источников антиоксидантов.

*Keywords:* lemon, antioxidants, lemon fruits, flavones.

*Ключевые слова:* лимон, антиоксиданты, плоды лимона, флавоны.

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Lipids and lipid-soluble substances that may be susceptible to oxidation are present in almost all foods, and lipid peroxidation plays an primary role in the deterioration of foods during storage. In addition, toxic substances formed by lipid peroxidation may lead to other adverse effects such as carcinogenesis, mutagenesis, and aging. The addition of antioxidants to food products (in fats and oils) is effectively helpful in retarding the oxidation of lipids and increasing shelf life and to reduce wastage and nutritional losses [1, 2].

Among the synthetic antioxidants, the most frequently used to preserve food are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have restricted use in foods because they are suspected of being carcinogenic. Some chemicals that occur naturally in plants have begun to receive considerable attention as safe antioxidants because they have been consumed by humans and animals for many years. Therefore, the search for natural antioxidants, especially those of plant origin, has greatly increased in recent years [6].

Oxidation in living organisms is essential for the acquisition of energy required to support biological processes. However, oxygen-centered free radicals and other reactive oxygen species (ROS) that are continuously produced in vivo can result in cell death and tissue damage. Oxidative damage caused by free radicals may be associated with aging and various diseases, such as atherosclerosis, diabetes, cancer, and cirrhosis. Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to completely prevent such damage. However, natural antioxidants derived from dietary plants have been reported to protect against oxidative damage caused by free radicals and reactive oxygen species and may heAlp prevent the development of diseases, cancer, and age-related disorders [10; 12].

It is widely accepted that fruits and vegetables possess numerous health-promoting properties. Citrus fruits, including oranges, lemons, limes, and grapefruits, are important sources of essential nutrients. They contain vitamin C, dietary fiber, and other bioactive components, such as carotenoids and flavonoids, which are suggested to contribute to the prevention of degenerative diseases. The lemon (*Citrus limon* (L.) Burm.f.) contains numerous important natural chemical constituents, including citric acid, ascorbic acid, minerals, and flavonoids. Numerous studies have shown that Citrus limon is a source of diverse nutrients and non-nutrient compounds, many of which exhibit bioactive properties. Examples of such non-nutrient compounds found in Citrus limon include organic acids and polyphenols. Organic acids are widely distributed in fruits and vegetables. In lemon, for instance, the seeds contain mainly eriocitrin and hesperidin, whereas the peel is rich in neoeriocitrin, naringin, and neohesperidin. Although the health-promoting properties of citrus fruits have traditionally been associated with their vitamin C content, recent studies have shown that flavonoids also contribute to these beneficial effects. Argall, Jealous, Lee, and Bradbury reported the major organic acids present in the leaves of Citrus limon. Phenolic compounds have been reported to possess multiple biological effects, including antioxidant, antitumor, antimutagenic, and antibacterial activities [9; 14].

Several studies have investigated the antioxidant activity of various citrus fruits, including sweet orange, lemon, and grapefruit, in food systems, either in their intact form or as extracts. However, limited information is available regarding the antioxidant activity of *Citrus limon*. To our knowledge, relatively little is known about the chemical constituents and antioxidant properties of *Citrus limon* leaves. Furthermore, the antioxidant potential of *Citrus limon* leaves has not yet been sufficiently investigated. Therefore, the objective of this study was to investigate the antioxidant and

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free radical-scavenging activities of extracts obtained from *Citrus* species, particularly *Citrus limon* leaves [7; 13].

The presence of various antioxidant compounds in plant tissues, particularly fruits and vegetables, makes it relatively difficult to measure each antioxidant component separately. Therefore, several methods have been developed in recent years to evaluate the antioxidant activity of biological samples. The antioxidant properties of *Citrus limon* leaf extracts were evaluated using several assays, including the ferric thiocyanate method, reducing power assay, DPPH radical-scavenging assay, and ferrous ion-chelating assay [1; 5].

*Materials and methods*

All chemicals used in this study were of at least analytical grade. Ethanol (99.7%) and hydrochloric acid (HCl) were purchased from Riedel-de Haën. Polyoxyethylene sorbitan monolaurate (Tween-20) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical were purchased from Fluka. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), butylated hydroxyanisole (BHA), phosphate buffer tablets (0.02 M, pH 7), Folin–Ciocalteu phenol reagent, ammonium thiocyanate ($NH_4SCN$), gallic acid, butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), (±)-α-tocopherol, linoleic acid, catechol, glacial acetic acid, and methanol were purchased from Sigma Chemical Co. (St. Louis, USA). L-ascorbic acid, ferrous chloride ($FeCl_2 \cdot 4H_2O$), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), and 4-dimethylaminocinnamaldehyde were obtained from Aldrich Co. EDTA was purchased from BDH Chemicals Ltd. [3; 6].

The plant leaves were thoroughly dried and powdered using a blender (Biohit). The air-dried leaf powder (10 g) was extracted with ether (500 mL) in a Soxhlet apparatus until the extraction solvent became colorless. The residue was then air-dried and extracted with ethanol (200 mL) for 10 h. Finally, the residue was air-dried and extracted with water (200 mL) for 10 h at a constant temperature of 25°C with continuous stirring. The extracts were filtered through Whatman No. 1 filter paper. The ether and ethanol extracts were evaporated at temperatures below 30°C using a Büchi RE 111 vacuum rotary evaporator (Büchi Laboratoriums-Technik, Flawil, Switzerland) under reduced pressure to remove the solvents. The aqueous extract was freeze-dried using a freeze dryer at a pressure of 5 mm Hg and a temperature of −50 °C. All extracts were stored under nitrogen at 4°C until further analysis. The extract solutions were prepared by dissolving 10 mg of each dried extract in 10 mL of the appropriate solvent. Ethanol was used as the solvent for both the ethanol and ether extracts, whereas water was used as the solvent for the dried aqueous extract. Although the same solvents were used for all assays, the concentrations differed among the assays, as described below. No detectable effect of the solvents on any of the measured activities was observed, as confirmed by control experiments in which solvents without extracts were used in the assays [4; 8].

The antioxidant activity of Citrus limon leaf extracts and standard compounds in inhibiting linoleic acid peroxidation was evaluated using the thiocyanate method. A 0.5 mL methanolic solution of the extracts (100–1000 mg) was mixed with linoleic acid emulsion (2.5 mL, 0.02 M, pH 7.0) and phosphate buffer (2 mL, 0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween-20 as an emulsifier, and 50 mL of phosphate buffer, followed by homogenization. The reaction mixture was incubated at 37 °C in a glass flask to accelerate the oxidation process. The peroxide value was determined by measuring the absorbance at 500 nm using a U-2000 spectrophotometer after reaction with $FeCl_2$ and thiocyanate at regular intervals during the 5-h incubation period. The inhibition of lipid peroxidation (%) was calculated using the following equation: % Inhibation = 1 – [(A1/A0) x 100].

Where $A_0$ represents the absorbance of the control reaction and $A_1$ represents the absorbance in the presence of the extract sample. The antioxidant activities of BHA and α-tocopherol were also evaluated at the same concentrations for comparison. A control reaction was performed using linoleic

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acid without the addition of the samples. Solutions without added extracts were used as blank samples. All data are presented as the mean of three independent analyses [9].

Total soluble phenolic compounds in the extracts were determined using the Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1977), with gallic acid used as the standard phenolic compound. Briefly, 0.2 mL of the extract solution, containing 100–1000 µg of extract, was mixed with 0.3 mL of 0.3% HCl. Then, 0.2 mL of this mixture was added to 4 mL of 2% $Na_2CO_3$ solution. After 3 min, 0.2 mL of Folin–Ciocalteu reagent was added, and the contents of the flask were mixed thoroughly. The mixture was then allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 760 nm using a UV–Vis spectrophotometer [12, 14].

The concentration of total phenolic compounds in the extracts was expressed as milligrams of gallic acid equivalents (GAE) using the equation obtained from the standard gallic acid calibration curve. The equation is given below. Absorbance = 0.0015 * gallic acid (µg/ml)-0.0243.

Total flavonoid content in the extracts was determined according to the method of Delcour and De Varebeke (1985), using catechol as the standard flavonoid compound. Briefly, 1 mL of the extract solution, containing 100–1000 µg of extract, was mixed with 5 mL of 0.1% 4-(dimethylamino) cinnamaldehyde reagent (HCl, 1:3). After 10 min, the absorbance was measured at 640 nm using a UV–Vis spectrophotometer. The total flavonoid content of the extracts was expressed as catechol equivalents (CE) using the equation obtained from the standard catechol calibration curve. The calibration equation was as follows: Absorbance = 0.0276 × catechol (µg/mL) + 0.0019. The experimental results are expressed as the mean ± standard deviation (SD) of five parallel measurements. P-values < 0.05 were considered statistically significant, while P-values < 0.01 were considered highly significant.

*Results and Discussion*

The presence of various antioxidant components in plants, particularly fruits and vegetables, makes it relatively difficult to determine each antioxidant component separately. Therefore, several methods have been developed in recent years to evaluate the antioxidant activity of biological samples. In the present study, the antioxidant properties of the samples were evaluated using several assays, including total antioxidant activity, DPPH radical-scavenging activity, ferrous-ion-chelating activity, and reducing power. Phenolic compounds are aromatic secondary metabolites of plants and are widely distributed throughout the plant kingdom. The total phenolic content (TPC) of Citrus limon leaf extracts was determined using the Folin–Ciocalteu phenol reagent. The Folin–Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of phenolic compounds present in plant extracts. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent. However, the assay has been shown to be non-specific to polyphenols alone, as other substances that can be oxidized by the Folin–Ciocalteu reagent may also contribute to the measured response. Various researchers have reported the limited specificity of this assay [3].

In addition, phenolic compounds respond differently to the Folin–Ciocalteu reagent depending on the number and nature of their phenolic groups [4].

Therefore, the Folin–Ciocalteu method is not actually an antioxidant assay; rather, it provides an estimate of the quantity of oxidizable substances, including phenolic compounds. As shown in Table 1, the total phenolic contents of the Citrus limon leaf extracts were in the following order: ethanol > ether > water. Although a significant difference (P < 0.05) in total phenolic content was observed between the ether and water extracts, the total phenolic content of the ethanol extract was slightly higher than that of the ether and water extracts. The total phenolic contents of the ether, ethanol, and water extracts were relatively high. Consequently, the ether, ethanol, and water extracts exhibited strong antioxidant activity (>90%) in the linoleic acid emulsion system (Table 1).

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Т. 12. №11 2026
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Table 1

ANTIOXIDANT ACTIVITY

| | *Amount yield %* | *Phenolic content, g* | *mg/mg extract* | *Flavonoid, µg/mg* |
|---|---|---|---|---|
| Ether extract | 3.86 | 7.72 | 136.0 | 1.96 |
| Ethanol extract | 2.33 | 4.66 | 152.0 | 9.56 |
| Water extract | 4.78 | 9.56 | 134.5 | 8.70 |

There are currently many methods available for determining antioxidant activity; moreover, each method can be used to elucidate the possible antioxidant mechanisms of the tested material. Therefore, extracts from Citrus limon leaves were evaluated for their total antioxidant activity, free-radical-scavenging activity, ferrous-ion-chelating capacity, and reducing power. In this study, the total antioxidant effects of ether, ethanol, and water extracts from Citrus limon leaves on the peroxidation of linoleic acid were determined using the ferric thiocyanate (FTC) method. The FTC method was used to measure the peroxide level during the initial stage of lipid oxidation. Lower absorbance values indicate higher antioxidant activity. In the FTC assay, the amount of peroxide formed during the initial stages of lipid oxidation was measured every 5 h over a period of 3 days. The absorbance of the positive control reached a maximum at 70 h. The absorbance of the control increased as a result of the oxidation of linoleic acid, which generated linoleic acid hydroperoxides. These hydroperoxides subsequently decomposed into various secondary oxidation products. During the FTC assay, lipid hydroperoxides react with ferrous ions, leading to the formation of ferric ions, which subsequently react with ammonium thiocyanate to produce a blood-red ferric thiocyanate complex. Antioxidants can inhibit or delay the peroxidation of linoleic acid; consequently, the formation of ferric thiocyanate is reduced, resulting in lower absorbance values. The antioxidant activities of ether, ethanol, and water extracts from Citrus limon leaves during the peroxidation of a linoleic acid emulsion system were compared with those of the commercial antioxidants BHA and α-tocopherol, as shown in Table 2. Significant differences ($P < 0.05$) were observed between the control and the samples containing the herbal water extracts, which slowed the rate of peroxide formation. As shown in Table 2, the ether and water extracts exhibited stronger antioxidant activity against linoleic acid peroxidation. The percentages of inhibition in the linoleic acid emulsion system for the crude ether, ethanol, and water extracts, BHT, and α-tocopherol at a concentration of 100 µg/mL were 98.1%, 79.4%, 99.9%, 77%, and [value to be confirmed], respectively.

Table 2

ANTİOXİDANT ACTİVİTY OF ETHER, ETHANOL, AND WATER EXTRACTS FROM LEAVES OF *Citrus limon (*LCL) İN THE LİNOLEİC ACİD EMULSİON PEROXİDATİON SYSTEM AS MEASURED BY THE THİOCYANATE METHOD AT 60 h

| *Sample* | *Inhibition of lipid per oxidation (%)*[a] | | | | | |
|---|---|---|---|---|---|---|
| | *100 µg/ml* | *200 µg/ml* | *400 µg/ml* | *600 µg/ml* | *800 µg/ml* | *1000 µg/ml* |
| EELCL (Ether) | 98.1 | 97.9 | 97.1 | 96.8 | 96.2 | 95.4 |
| EtELCL (CL Ethanol) | 79.4 | 100 | 100 | 100 | 100 | 100 |
| WELCL (water) | 99.9 | 99.5 | 99.2 | 99.0 | 99.1 | 93.7 |

[a]The peroxide values were determined by measuring the absorbance at 500 nm after reaction with $FeCl_2$ and thiocyanate; [b]Each value is mean_S.D. (n=3)

At a concentration of 0.2 mg/mL, all extracts exhibited excellent antioxidant activity, with inhibition values ranging from 97.9% to 100%. As shown in Table 2, the percentages of inhibition at a concentration of 1000 µg/mL for the ether, ethanol, and water extracts in the linoleic acid emulsion system were 95.4%, 100%, and 94.6%, respectively.

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These values were slightly higher than that obtained for 0.2 mg/mL BHA (93.8%) but were considerably higher than that obtained for 0.2 mg/mL α-tocopherol (72.4%), indicating that antioxidant activity was closely related to the concentration of the extracts. The antioxidant activities of the ether and water extracts of Citrus limon leaves at a concentration of 0.1 mg/mL were slightly lower than that of BHA but higher than that of α-tocopherol. These results indicate that the ether and water extracts of Citrus limon leaves possess strong antioxidant activity and are effective in inhibiting linoleic acid peroxidation.

In general, *Citrus limon* leaves may play an important role in protecting against oxidative damage because they contain endogenous antioxidants, including phenolic compounds and other phytochemicals. The main purpose of using antioxidants is to prolong the induction period of autoxidation and, consequently, improve oil stability. It is evident that the addition of all the extracts significantly prolonged the induction period of linoleic acid oxidation, as indicated by the lower rate of accumulation of oxidative products compared with the commercial antioxidants. It has long been recognized that naturally occurring substances in higher plants possess antioxidant activity. Because of the complex composition of plant extracts, their antioxidant activities cannot be evaluated using a single method alone. In the present study, we evaluated the total antioxidant activity of ether, ethanol, and water extracts from Citrus limon leaves and compared their activities with those of the standard antioxidants BHA, BHT, and α-tocopherol.

In the present study, the total antioxidant activities of the ether, ethanol, and water extracts of Citrus limon leaves were determined using the thiocyanate method. In this method, the amount of peroxides formed in the emulsion during incubation is determined spectrophotometrically by measuring the absorbance at 500 nm. High absorbance indicates a high concentration of formed peroxides, whereas low absorbance indicates higher antioxidant activity. The stable free radical DPPH has been widely used to determine the primary antioxidant activity of Citrus limon extracts. The assay is based on the reduction of DPPH radicals in ethanol, which results in a decrease in absorbance at 517 nm.

## *Conclusion*

Antioxidants can exert their protective effects at different stages of the oxidation process and through different mechanisms. Furthermore, the complex composition of plant extracts may be responsible for various interactions, including synergistic, additive, or antagonistic effects, among their constituents or between the constituents and the reaction medium. The relationship between the antioxidant or free-radical-scavenging activity of a plant extract and its phenolic content is difficult to establish using statistical analysis. This is because the antioxidant properties of individual compounds within the same group can vary considerably, while the different methods used to determine antioxidant activity are based on different reaction mechanisms. Furthermore, plant extracts are complex mixtures containing compounds with different polarities and antioxidant or prooxidant properties, which may sometimes exhibit synergistic effects compared with individual compounds. *The Citrus limon* leaf extracts prepared using water as the solvent were also highly active, despite containing lower levels of detected phenolic compounds. This finding suggests that compounds other than phenolic constituents may contribute substantially to the antioxidant activity of the aqueous extracts.

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