Total Phenolic, Total Flavonoid Contents, and Antioxidant Activity of Fruit *Zizyphus Lotus* Extract

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Abstract

The purpose of this study was to evaluate, by a chemical method, screen various solvent extracts of the fruit *Zizyphus lotus* to display potent antioxidant activity, and total phenolic and flavonoid contents to find possible sources for future novel antioxidants in food and pharmaceutical formulations.

The method used to measure the antioxidant activity was free radical scavenging by using DPPH (1-1-diphenyl-2-picryl hydrazyl). The content of total phenols was determined using Folin-Ciocalteu reagent, whereas the Aluminum chloride colorimetric method was used for flavonoid determination.

Flavonoids and phenol contents varied according to the nature of the extract. The total phenolic contents, highest total phenolic content was recorded with Ethyl acetate extract (28.147 ± 0.649 mg GAE/g extract), whereas Butanol extract (8.630 ± 0.491 mg GAE/g extract) contained the lowest content of phenols. The highest total flavonoid content was found in Butanol extract (1.611 ± 0.138 mg QE/g extract). Meanwhile, the Ethyl acetate extract (1.302 ± 0.268 mg QE/g extract).

The Ethyl acetate extract showed the highest antioxidant capacity 0.331 mg/ml. the Butanol extract had the lowest antioxidant capacity 0.737 mg/ml.

These fruit *Zizyphus lotus* showed significant antioxidant activity, and flavonoid and phenolic contents, this may justify the use of *Zizyphus lotus* in traditional medicine. the result of that is the need to use the anti-oxidant of this plant as a rich source of antioxidants in food and pharmaceutical.

Keywords: Antioxidant activity, total phenolic and flavonoid contents, Folin-Ciocalteu, Aluminum chloride colorimetric, DPPH. *Zizyphus lotus*.

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Introduction

The study of plant chemistry is still of Great importance because the plant kingdom represents a source of bioactive molecules. Plants have many active compounds, they are the richest bio-resource of drugs of traditional and modern medicine (Hammar et al., 1999), this medicinal value is based on some chemical substances that produce a definite physiological action on the human body. [1]

Algeria is one of the richest countries with plants because of its vast area and climate diversity, but still, there are many species of plants that few studies are caring about them, so in this study, we chose one of these plants, which is *Zizyphus lotus*. This plant is a traditional medicinal plant Zizyphus (family of Rhamnaceae) is widespread in tropical and sub-tropical regions such Asia, Africa, North America, South America, Oceania, and Europe. There are several species of this genus (Ziziphus Vulgaris Lam, Ziziphus lotus Lam, Ziziphus Spina-Christi (L.) Wild, and Ziziphus mauritiana Lam), depending on the soil and climate [2].

Botanically, it belongs to the angiosperm Rhamnaceae family which contains about 135–170 species . The North African species of the genus Zizyphus are known by the vernacular names sedra,
addhal, çder nabga, dhou achaouk, and sder alberri. In Algeria Ziziphus lotus is known as "Sedra" and the fruit is called “Nbag”. Zizyphus lotus is used in the traditional therapeutic and nutrition, health, .and treatment of diarrhea and intestinal diseases. skin infections, fever, diarrhea, insomnia, sedation, bronchitis, and urolithiasis , Cyclopeptides alkaloids.

These biological and pharmaceutical properties of *Zizyphus lotus* are largely due to the presence of active substances such as flavonoids which represent one of the largest classes of natural products synthesized by the plant. this family has been exploited in the field of phytotherapy due to its multiple therapeutic properties. the most important activity of these compounds is antioxidant activity. [3]

Oxidation is a chemical reaction that transfers electrons or hydrogen from substances to an oxidizing agent. It can produce free radicals. These radicals can start a chain reaction when the chain reactions occur in a cell it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibiting other oxidative reactions. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. this leads to aging and to degenerative diseases of aging such as cardiovascular disease, cataracts, cancer, brain dysfunction, and immune system decline. drugs, pollution, stress, even exercise, cigarette smoke, and illness can increase free radicals. The need

for antioxidants becomes even more critical because are our first line of defense against free radicals. [4,5]

It is in this sense that the study of the antioxidant activity of plants is important because we can find in plants powerful antioxidants. The purpose of this study was to evaluate the antioxidant activity, and phenolic and flavonoid contents of the fruit *Zizyphus lotus* in southeastern Algeria.

1. Materials and Methods

1.1. Preparation of Extract

The collected plant material was dried at room temperature and protected from light and humidity. After drying, fruits are crushed using an electric grinder until a powder is obtained. The fruits of the plant were soaked in a volume of 1 liter of solution (water/ethanol) at a ratio of (30/70) and left for 48 hours with shaking at room temperature. then the solution was filtered and concentrated under low pressure using a rotary evaporator to get rid of the ethanol. repeat the process 3 times until the concentration of the color of the filtrate weakens, that is, extracting the largest possible amount of active substances.

After the end of the filter evaporation process, the extract was obtained. we added distilled water and left for 24 hours, then filtered twice to remove impurities and we got the water phase. then selective extraction using solvents of increasing polarity from petroleum ether, chloroform, ethyl acetate, and, butanol. the solvent used was evaporated in each organic phase.

1.2. Chemical detection of some secondary metabolites

- **The general test for flavonoids**

A weigh of 10 g is taken from the dry plant and soak in 150 ml of Dilute (1%) hydrochloric acid for 42 h then filter. and add drops of ammonia is added to 10 ml of the obtained filtrate where the pH of the medium is followed by pH paper, after the basicity of the medium, we notice the appearance of the light-yellow color testifies to the presence of flavonoids.

- **Free flavonoids**

In a tube 5 ml of the resulting filtrate is introducing and adding 5.2 ml of Amyl alcohol (we note after Stir and equilibrating the alcohol phase (upper phase) is yellow, indicating on the presence of free flavonoids.

- **Test for glycosidic flavonoids**

Two methods for detecting glycosylated flavonoids are found:

✓ **First method:** The alcoholic phase obtained from the previous test (the detection of free flavonoids) was evaporated under pressure and the obtained precipitate was dissolved in 3 ml of dilute (1%) chlorhydric acid. After shaking and equilibrium, we notice the yellow coloration of the alcohol phase, evidence of the presence of glycosidic flavonoids.

✓ **Second method:** 5ml of the obtained filtrate is putting in a test tube and a small amount of magnesium (Mg) is added. We shake well. After a while, we notice the appearance of red color, which indicates the presence of glycosidic flavonoids.

- **Detection of other active ingredients in the plant**

✓ **Alkaloids test**

Weigh 10 g of dry vegetable powder, soak it in 150 ml of dilute chlorohydric acid (1%) for 48 hours and then filter, the obtained filtrate is titrated by ammonia solution NH_4OH (2N) until (pH=9) after the titration process, we perform the extraction process (liquid-liquid) three times by Chloroform. The organic phase is collected and evaporated by a rotary evaporator. The obtained precipitate is dissolved in 2 ml of dilute chlorohydric acid (1%) and three drops of Mayer's reagent. The formation of a white precipitate is observed, evidence of the presence of alkaloids

✓ **Cardénolides test**

Weigh 10 g of dry vegetable powder then soak it in distilled water for 20-30 minutes. We filter the extraction process (liquid-liquid) for the solution obtained by 10 ml of a mixture of chloroform and ethanol. The obtained organic phase is evaporated, and the resulting precipitate is dissolved in 3 ml of Glacial acetic acid to which drops of iron trichloride ($FeCl_3$) were added, followed by the addition of drops of sulfuric acid (H_2SO_4), and we notice the coloration of the acid phase in a bluish-green color, which indicates the presence of cardenolides.

✓ **Tannins test**

Weigh 10 g of vegetable powder, soak it in ethylene alcohol (50%) for 30 minutes, and filter, the obtained filtrate is added to it drops of iron trichloride After a while, we notice the appearance of green color, evidence of the presence of tannins

✓ **Test for unsaturated sterols and terpenes**

We weigh 15g of dry vegetable powder. Soak it in 20 ml of chloroform for 30 minutes, then filtered. 1 ml of sulfuric acid (H_2SO_4) were added to the obtained filtrate. With caution on the wall of the tube, we notice then the appearance of a green color that turns after a while to read in the separating layer between the two phases. This is evidence of the presence of unsaturated sterols and terpenes.

✓ **Saponin test**

Weigh 2 g of dry vegetable powder in 80ml of distilled water and heat for 15 minutes, then filter and cool. The filtrate is placed in a test tube and shaken well. It is left for a certain period. We note the appearance of a foam that remains for 15 minutes, evidence of the presence of saponin

✓ **Steroids test**

Weigh 5 g of vegetable powder, and soak in 20ml of ethylene alcohol (70%) for 30 minutes. The filter is evaporated. The obtained precipitate is dissolved in 20ml of chloroform. It is filtered again to get rid of impurities, so we get a filter that is divided into two parts:

- The section is placed in a test tube to which 1 ml of acetic acid (CH_3COOH) and 1ml of sulfuric acid (H_2SO_4), in case the color does not appear.
- The second section is placed in a test tube to which an equal volume of sulfuric acid is added to the wall of the tube. The appearance of the yellow color, which then turns red, is evidence of the presence of steroid derivatives.

- **Determination of total Phenolic**

The total amount of phenolic compounds was estimated using the cioalteu-folin reagent in an alkaline medium, and the number of phenols was estimated by measuring the absorbance of the samples using the UV spectroscopy apparatus at a wavelength of 760 nm. Acid gallic is used as the standard phenol.

Standard curve :

- We prepared solutions of different concentrations of gallic acid, ranging between (0.03-0.3) g / l.
- Take 0.1 ml of each concentration and add 0.5 ml of cioalteu-folin reagent, diluted 10 times. (Leave it for 5 minutes, then add 2ml of 20% sodium carbonate, and repeat 3 times we leave it for 30 minutes in the dark and at room temperature.
- We treated the butanol and acetate extract with the same treatment as gallic acid.

- **Determination of total Flavonoids**

The number of total flavonoids is estimated using aluminum chloride, which forms complexes with the phenolic compounds. Aluminum chloride is absorbed in the visible field at the wavelength of 430 nm, we used quercetin reference flavonoid.

Standard curve :

- Different concentrations of quercetin were prepared, between (0.01-0.1) g / l.
- 1.5 ml of 2% aluminum ethanol solution was added to 1.5 ml of each concentration and turn it 3 times, then left away from light for 30 minutes at room temperature. The absorbance is measured at Each concentration at 430 nm.
- The same steps were repeated for the butanolic and acetate extract.

- **DPPH radical scavenging activity method**

DPPH free radical is widely used to study the structure antioxidant activity relationship of phenolic compounds. DPPH has an uncured electron which allows the characteristic blue-violet color of the solution of DPPH molecules. Contact of DPPH with oxidizing molecules results in discoloration which thus marks the anti-radical capacity of the compound studied. [5] In our study, we chose to use ascorbic acid as a baseline in the capture of free radicals. Preparation of different concentrations of ascorbic acid between (0.01-0.1) g/l. We take 0.1 ml of each solution and 2.66 ml of the DPPH solution is added. Shake well and leave it in the dark for 30 minutes. The absorbance was measured at a wavelength of 517 nm, then through the product, the percentage of inhibition I% is calculated. This is according to the following relation:

$$\text{Inhibition (I\%)} = \frac{(\text{Absorbance control} - \text{Absorbance sample})}{\text{Absorbance control}} \times 100$$

✓ **Determination of IC50 values**

IC50 values were calculated from the plotted scavenging activity against the concentrations of the samples. IC50 is defined as the total antioxidant necessary to decrease the initial DPPH radical by 50 %. the lower the EC50 of a compound, the more antioxidant capacity it has.

2.Results and discussion

Phytochemical screening was carried out to determine the secondary metabolites in the sample. The result of the phytochemical screening assay can be seen in Table 1.

Table 1: Results of the initial chemical tests for the fruit *Zizyphus lotus* plant.

Active ingredient	Percentage of presence
Glycoside Flavonoids	++
Flavonoids	+++
Free Flavonoids	+++
Alkaloids	+++
Cardenolides	+
Tannins	+++
Saponoids	+++
Steroids	-
Unsaturated sterols and terpenes	+
Unsaturated steroids	++

+++: Large percentage.
+: Small percentage.
++: Average ratio.
-: Not being present.

Phytochemical screening was carried out due to its simplicity, speed, minimum use of equipment, and selectivity. allows us to have a good idea of the phytochemistry of the medicinal plant. The results obtained confirmed the presence of several phytochemicals (bioactive compounds), that have a crucial role in biological activity.

Flavonoids, free flavonoids, tannins, alkaloids and Saponoids in the medicinal plants studied are present in large quantities, in addition to the presence of unsaturated sterols, terpenes, and cardenolides in small quantities, as for steroids, it is noted that they are absent in the plant. could justify the traditional use of *Zizyphus lotus*.

2.1. Determination of total phenolic contents

Figure 1 shows the gallic acid calibration curve obtained for the assay of total phenols, as determined by folin Ciocalteu method, The linearity of the calibration curve has been tested at concentration ranges (0.03 - 0.3 mg/ml) gallic acid as standard solutions of total phenolic

content, the equation obtained from the linear calibration graph of the concentrations studied: y = 3.429x +0.012 (R^2 = 0,990).

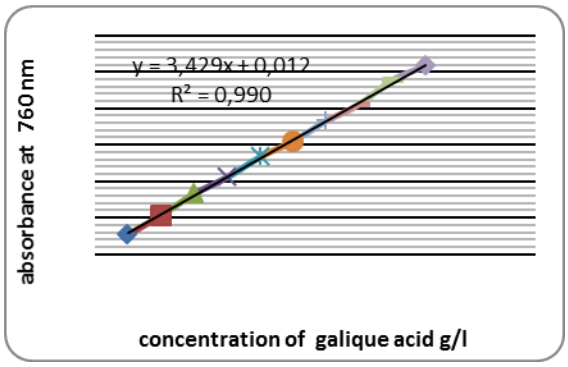


Figure 1 : Calibration curve of standard gallic acid

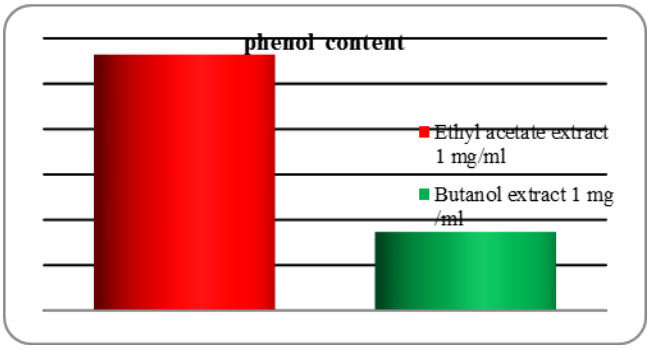


Figure 2 : Content of phenol for Ethyl acetate for determination of total phenolics. extract and Butanol extract.

Concerning phenol contents varied among the samples and ranged highest phenol contents were noted in Ethyl acetate extract (28.147 ± 0.649 mg GAE/g extract), whereas Butanol extract (8.630 ± 0.491 mg GAE/g extract) contained phenols contents.

- **Determination of total flavonoids**

The linearity of the regression line of the calibration curve was evaluated by preparing standard solutions of quercetin in ethanol at concentration ranges of 0.01 to 0.1 mg/ml for total flavonoid content. The equation obtained from the linear calibration plot of concentrations studied y = 29,40x + 0,359, R^2 = 0,944.

Where **y** represents the absorbance value and X is the Quercetin concentration value (Figure 3).

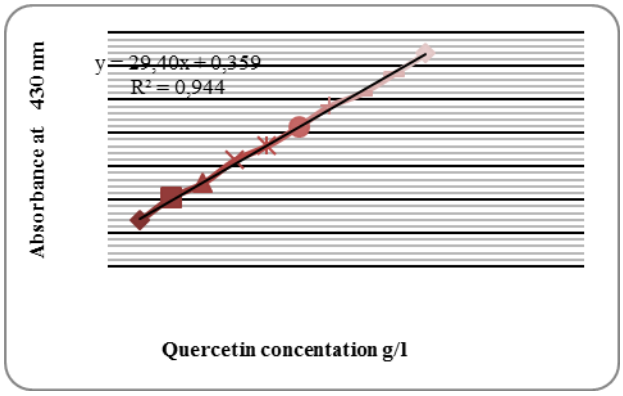


Figure 3: Calibration curve of standard Quercetin

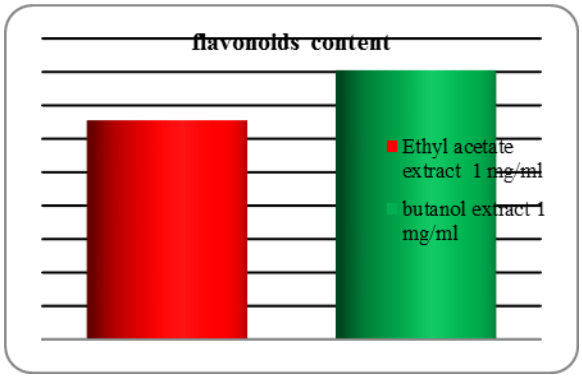


Figure 4: Content of flavonoids for Ethyl for determination of total flavonoid content acetate extract and Butanol extract

Total flavonoid contents in the Butanol extract were (1.611 ± 0.138 mg QE/g extract) and (1.302 ± 0.268 mg QE/g extract) in the Ethyl acetate extract. The measurement results of absorbance of the Ethyl acetate extract and Butanol extract, which were plotted on gallic acid curves and quercetin curves showed different levels of total phenols and total flavonoids as listed in the following table 2:

Table 2: phenol and flavonoids content of Ethyl acetate extract and Butanol extract

Extract	Phenol content (mg GAE/g extract)	Flavonoids content (mg QE/g extract)
Ethyl acetate extract	28.147 ± 0.649	1.302 ± 0.268
Butanol extract	8.630 ± 0.491	1.611 ± 0.138

The results of the assay reveal that the Butanol extract is the richest in flavonoids, on the other hand, the Ethyl acetate extract is the richest in phenols in total. the difference in the total contents of phenolic and flavonoids may be due to differences in the geographical region,

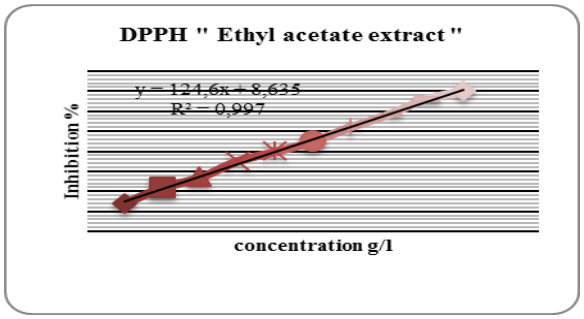
climatic condition, environment, degree of maturation of the plant, storage, and processing methods [20, 21].

Antioxidant activity

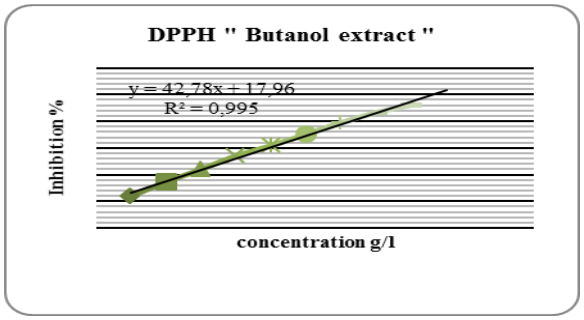
Application of antiradical activity:

The antioxidant capacity can be tested using a wide variety of methods. In the present study, the antioxidant activity of the extracts of *Zizyphus lotus* was evaluated in terms of their free radical scavenging capacity by the DPPH assay. The DPPH, a purple-colored free radical is reduced to a yellow-colored compound in the presence of scavenging compounds. The intensity of the coloration, measured with a spectrophotometer, is inversely proportional to the antiradical activity of the various extracts whose activity one wishes to determine.

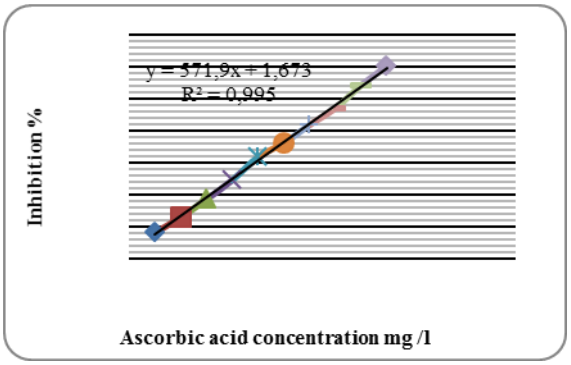
Figure 5: shows the percentage inhibition of the DPPH radical as a function of the concentration of Ethyl acetate extract and Butanol extract.



(a)



(b)



(c)

Figure 5: (a, b) Scavenging effect of DPPH by ethyl acetate extract and butanol extract, respectively. **(c)** Calibration curve for ascorbic acid (vitamin C).

The antioxidant activity of the standard (ascorbic acid) or extracts was expressed as the concentration of the extract or the standard providing 50 % inhibition (IC50), this concentration was determined graphically by plotting to show the percentage inhibition against the extract concentration. IC50 for DPPH radical -scavenging activity are shown in figure 7.

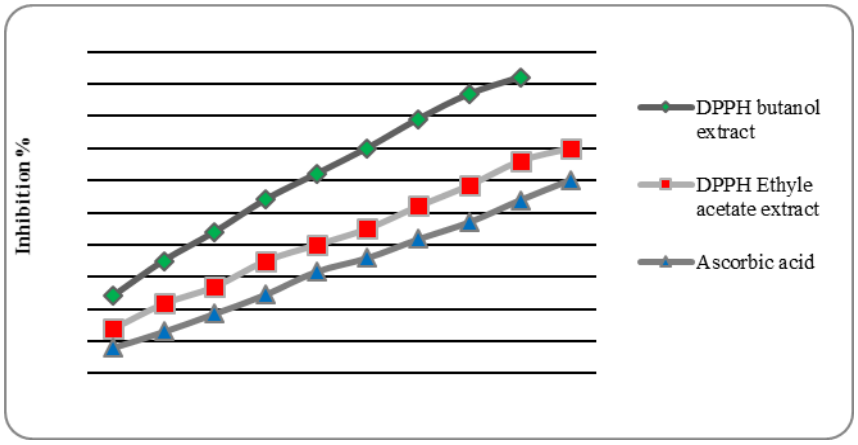


Figure 6: DPPH radical scavenging activity Ethyl acetate extract, Butanol extract, and Ascorbic acid.

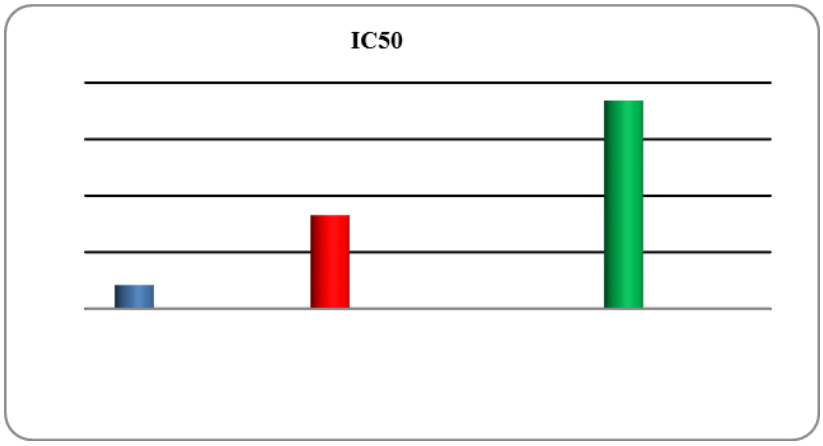


Figure 7: IC50 of Ascorbic acid, Ethyl acetate extract, and Butanol extract.

The antioxidant activity of two extracts from the fruit *Zizyphus lotus* is expressed in terms of the percentage of inhibition (%) and IC50 values (mg/ml). The values for the standard compound were obtained and compared to the values of the antioxidant activity. The examination of the antioxidant activities of plant extracts showed different values. The largest capacity to neutralize DPPH radicals was found for Ethyl acetate extract, which neutralized 70 % of free radicals at the concentration of 0.331 mg/ml. Moderate activity was found for Butanol extract 92% at the concentration of 0.737 mg/ml.

Most plant species that grow around the world have therapeutic virtues because to produce diverse substances possessing antioxidants. the roles of antioxidants are to neutralize the excess of free radicals, protect the cells against their toxic effects and contribute to disease prevention. Based on the results of these antioxidant activity assays, phenolic and flavonoid

content determination indicates that there is a positive relationship between total phenol content and radical scavenging activity. Ethyl acetate extract contains the highest amounts of phenolics and has a high level of antioxidant activity.

Phenols are very important plant constituents, these compounds are a class of antioxidant agents that act as free radical terminators, because of the scavenging ability of their phenolic hydroxyl groups. The antioxidant capacity of phenolic compounds depends on the number and position of free OH groups, which means, that the freer hydroxyl groups present in phenols, the higher their radical scavenging capacity [6]. phenolic compounds are also reported to be effective hydrogen donors, making them very good antioxidants [7].

The antioxidant activity of medicinal plants could be attributed to its flavonoids content. It has been recognized that flavonoids are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities and their effects on human nutrition and health are considerable (van Acker *et al.*, 1996). the mechanisms of action of flavonoids are through scavenging or chelating process flavonoids act as scavengers of various oxidizing species superoxide anion, hydroxyl radicals, or peroxy radicals, they also act as quenchers of singlet oxygen [8]. Moreover, flavonoids may exert their cell structure protection through a variety of mechanisms; one of their potent effects may be through their ability to increase levels of glutathione, a powerful antioxidant, as suggested by various research studies.[9]

There is a specific relationship between flavonoid structures and their antioxidant activity as the larger the number of hydroxyl groups in the flavonoid's nucleus, the greater would be the antioxidant activity [22]. This reinforced the idea that the antioxidant potential could be linked strongly to the content of flavonoids in the plant.

Conclusion

The results obtained confirmed the presence of several phytochemicals (bioactive compounds), having a crucial role in biological activity such as phenols, flavonoids, tannins, and alkaloids, which could justify the traditional use of *Zizyphus lotus.* The results of the present study suggested that the extracts of fruit the Zizyphus *lotus* could be a potent source of natural antioxidants because of their phenolic and flavonoid contents and their remarkable scavenging effects on DPPH. Therefore, it can be considered a potent natural composition requiring a high valorization of this by-product using it as an ingredient to enhance some functional foods' nutritional value for human and animal consumption. This medicinal plant by in vitro results appears interesting and promising and may be effective as a potential source of novel antioxidant drugs and cosmetic and natural oils.

The advantages of using natural antioxidants, particularly combinations with synergistic outcomes, should be taken seriously; systematic investigations into the multifaceted interactions among natural and synthetic antioxidants, and with different food matrices, must be pursued.

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