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TOTAL PHENOLIC AND FLAVONOID CONTENT, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF *ZIZIPHUS LOTUS* FROM MOROCCO

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Abstract

In this study, total phenolic and flavonoid content as well as in vitro antioxidant and antimicrobial activity of the methanolic extract of *Ziziphus lotus* were evaluated using different methods. The total phenol and flavonoid content in the extract were determined by Folin Ciocalteu and AlCl3 assays, while antioxidant and antibacterial activities were studied using DPPH free radical-scavenging, ABTS, disc diffusion and micro-dilution methods. The results showed that the phenols amount (143.12 mg/mL) in the methanolic extract of *Ziziphus lotus* were thirty-five fold higher than the flavonoids (4.28 mg/mL). Furthermore, *Ziziphus lotus* showed higher antioxidant potency as determined by DPPH and ABTS methods, (DPPH IC50 values of 131.01 µg/mL compared to 50.67 for BHT, and ABTS IC50 values of 52.42 µg/mL compared to 63.44 µg/mL for Trolox). Accordingly, antibacterial tests showed that the methanolic extract exhibited a powerful antibacterial effect against the bacterial strains with MIC values of 400 and 320 µg/ml against E.coli and Bacillus pumilus, respectively. These results indicated that *Ziziphus lotus* fruit could be taken as a potential source of phenolic compounds well-known for their impact in human health as well as nutrition.

Keywords: *Ziziphus lotus, antibacterial, antioxidant, phenols, flavonoids*

Introduction

An antioxidant is considered to be any chemical substance that prevents the oxidation of a substrate in presence of an oxidisable compound [1]. Polyphenols, carotenoid and traditional antioxidant vitamins such as vitamin C and E, are considered among the major phytochemicals liable to the antioxidant activity in plant materials. Phenolic substances have been studied rigorously for their human health benefits, and have been considered the most bioactive phytochemicals for this purpose [2]. Their antioxidant power is mainly due to their redox properties of the phenolic hydroxyl groups and the chemical structure [3–5]. It has been proven that antioxidant substances are associated with many biological benefits, such as the maintenance of the immune functions, the diminution of lipid peroxidation and DNA damages [6]. Because of their ability to scavenge free radicals, antioxidant substances have attracted great interest [7]. Many disorders in human organism (e.g., neurodegeneration, Alzheimer disease, cancer and inflammation) may result from increased concentrations of these free radicals in organisms [8–10] Furthermore, the antioxidant substances probably prevent human organism against several diseases, for instance, ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders [11].

Accordingly, natural antioxidants present in plants have no side effects, whereas chemically synthetized antioxidants were found to have multiple genotoxic effects [12,13]. It is the main reason behind the consistent and great effort that the researchers put into finding new natural antioxidants and thus, safe, effective and cheap alternatives to the synthetic products [14].

Ziziphus lotus (*Rhamnaceae*) is a widely spread fruit in the Mediterranean zone. It is used in traditional medicine to treat sore throats, alleviate stress and helps common colds [15]. *Ziziphus lotus* is also claimed to purify blood, help digestion and diet [16]. The most present metabolites in the Ziziphus genus are the phenolic substances. Several metabolites, namely flavonoids, tannins and alkaloids showed great pharmacological properties [17]. These metabolites are widespread in plants where they act as antioxidants and free radical scavenger [18–20]. Previous studies showed strong relationship between total phenolic content and antioxidant activity in different seeds, fruits and greens [21]. The main aim of this study was to determine the total phenolic (TPC) and flavonoid (TFC) content, the antibacterial and antioxidant activity (AA) of the *Ziziphus lotus* fruit as well as their correlations.

Methods

Chemicals

Rutin, Trolox, Folin-Ciocalteu reagent, Butylated hydroxytoluene (BHT) , 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and Potassium persulfate were all obtained from Sigma. Sodium nitrite, Aluminium chloride and Sodium carbonate were from Loba Chemie. Gallic Acid was from Panreac. All the solvents were of analytical grade and were used without further purification.

Collection and preparation of extracts

Ziziphus lotus fruit used in this study was collected from Zaouiat Cheikh area, near Oued Zem City, Morocco. The fruit was air-dried, blended and powdered afterwards. Dried fruit (30 g) was extracted by cold maceration with pure water and methanol (200 mL) separately at room temperature. The extracts were filtrated three times and concentrated using rotary vacuum evaporator (Heidolph G 1, Germany) at 40-50°C under vacuum and dried residues were collected into Eppendorf tubes and stored at – 4°C for experimental use.

Antibacterial activity

Extracts were tested against the clinically pathogenic gram (-) bacteria (e.g., Escherichia coli, Agrobacterium sp., and Rhizobium sp.) and gram (+) bacteria (e.g., Bacillus pumilus and bacillus subtilis). Antibacterial activity was determined using agar well diffusion assay according to NCCLS [22]. Petri plates containing 20 ml broth agar medium were seeded with bacterial strains for 24 h. Wells (6 mm diameter) were cut into the agar and 10 µl of *Ziziphus lotus* extracts were tested in a concentration 94.28 and 3.03 mg/mL for the water and methanol extracts respectively. The plates were then incubated at 28°C for 24 hours. The inoculum size was adjusted to

deliver a final inoculum ~107 colony-forming units (CFU/mL). The antibacterial activity was assayed by measuring the diameter of the inhibition area formed around the well. Minimum inhibitory concentration (MIC) was determined by the micro-dilution method using serially diluted *Ziziphus lotus* extracts (2-fold) according to NCCLS (Cockerill et al. 2012). Both aqueous and organic *Ziziphus lotus* extracts were tested. Bacterial inoculums were adjusted to contain approximately 107 CFU/mL.

Determination of total phenolic content

The total phenolic content TPC of *Ziziphus lotus* extract was determined using Folin-Ciocateu [24]. To an aliquot (0.5 mL) of extracts, 2.5 mL Folin-Ciocalteu reagent diluted with H_2O to a ratio of 1:10 was added , then 4 mL of Na_2CO_3 (7.5 %, w/v) was added, and mixed thoroughly. The mixture was then incubated at 45°C for 30 min, and the absorbances were taken against a blank at 765 nm. TPC was expressed as mg Gallic Acid equivalents per 100g dry weight (mg GAE/100g dw) using freshly prepared Gallic Acid solution as reference.

Determination of flavonoids content

The total flavonoid content TFC was determined using aluminium chloride colorimetric method [25]. To an aliquot (1 mL) of extracts, H_2O was added to a 5 mL volume; 0.3mL of $NaNO_2$ was then added. After 5 min, 0.3mL $AlCl_3$ was added and well shaked. At the 6th min, 2 mL of a 1M NaOH was added and the absorbance was measured against reagent blank at 510 nm. TFC was expressed as Rutin equivalent (mg RE/100g dw) fresh mass using Rutin as reference.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay

The antioxidant activity of *Ziziphus lotus* extract was determined using 1,1-diphenyl-2-picryl-hydrazyl test with small modifications [26]. Concisely, in a test tube, 0.5 mL of DPPH (0.2 mM) was mixed with an aliquot (2.5mL) of plant extract, and incubated at room temperature for 30 minutes. The absorbance was measured against blank samples at 517 nm. High free radical scavenging activity is equivalent to low absorbance of the reaction mixture. The percentage scavenging activity was determined using Eq.1:

$$\text{RSA}\ (\%) = \frac{\text{AD} - \text{AE}}{\text{AD}} \text{ x } 100 \quad (1)$$

Where AD is the absorbance of the DPPH blank sample, and AE is the absorbance of the test solution. AE was calculated as the difference between the absorbance of the test solution and its blank. The results were compared to those of butylhydroxytoluene (BHT).

ABTS radical scavenging assay

The ABTS•+ decolorization assay was done as described by [27] with small modifications. Mixing solution of ABTS (2mM) and $K_2S_2O_8$ (70 mM) with 1:1 ratio was incubated in dark for 24 h at room temperature to generate deep colored radical containing solution. Then, 2 mL of the resulting solution was added to 200µL of *Ziziphus lotus* extract with different concentrations. The absorbance was measured at 734 nm after 30 minutes and the inhibition percentage was calculated using Eq. 2:

$$\text{ABTS}\ (\%) = \frac{1 - \text{Abs sample}}{\text{Abs Blank}} \text{x } 100 \quad (2)$$

Where Abs blank is the absorbance of the blank sample, and Abs sample is the absorbance of test sample. The obtained results were in line with those of Trolox.

Results and discussion

Antibacterial activity

The antibacterial proprieties of *Ziziphus lotus* extracts in aqueous and organic solutions were evaluated using disc diffusion method as well as micro-dilution method against different microorganisms (e.g., E.Coli, Agrobecterium sp, Rhizobium sp, Bacillus pumilus and Bacillus subtilis) and the results are shown in Table 1. These methods were used to test the susceptibility of bacteria to the *Ziziphus lotus* extracts. It seems that the microorganisms tested in this study were not as sensitive to aqueous solution as compared to organic solution. In line with previous study where no inhibitory effect was observed in aqueous extracts [28,29]. Furthermore, the results showed that the *Ziziphus lotus* extracts were more active towards the Gram-positive bacteria. This was not surprising since Gram-negative bacteria are more resistant to plant extracts as compared to Gram-positive bacteria [29]. Such behavior could be explained by the permeability barrier provided by the cell wall or to the membrane

accumulation mechanism that is more effective in gram negative bacteria due to the presence of the outer membrane in their structure in contrast to gram positive bacteria [30].

Accordingly, the largest zone of inhibition was recorded for the methanolic extract against Rhizobium sp. with a diameter of 16 mm, followed by Bacillus pumilus, Agrobacterium sp., E.coli and Bacillus subtillis with diameters of 12, 11, 11 and 7 mm respectively. The most potent inhibition with the micro-dilution method was observed in case of Rhizobium sp with a MIC=3.2 µg/mL. It has been reported that antimicrobial activities of Rhamnacea species are mainly attributed to its most active ingredients (i.e., polyphenols and alkaloids) [15,31–33]. In this study, the water extracts were discarded in the micro-dilution method since they haven't shown any inhibitory effect in the disc diffusion method.

Total phenolic and total flavonoid content

The total phenolic content of the extracts were determined using Folin-Ciocalteu (FC) colorimetric method. This method allows the estimation of all flavonoids, anthocyanins and other phenolics compounds (non-flavonoids) present in the extracts. The total flavonoid content was assessed by precipitating the crude extract with aluminum chloride ($AlCl_3$), allowing Al^{3+} to bind with the ketone and hydroxyl group of the flavonoids through electron transfer reaction. A yellow colour is then observed under UV spectrophotometer at the maximum absorbance of 510 nm [34]. Figure 1 shows the TPC and TFC results of the methanolic extract of *Ziziphus lotus* and the calculated values of TPC and TFC were 143.12 mg/mL GAE and from 4.281 mg/mL RE, respectively. It has been reported that hydroxyls included in flavonoids has direct relationship with the radical scavenging effect, while phenols inhibits action of reactive oxygen species in the plants [19,35–37]. In addition, flavonoids are shared constituents of phenolics mainly synthetized by the phenylpropanoid metabolic pathway [38]. Accordingly, each of phenolics and flavonoids contribute widely to human health by their antioxidant and anticancer properties [39].

Antioxidant activities

Phytochemical diversity of secondary metabolites including alkaloids, terpenoids, phenols and flavonoids present antioxidant activity because of their red/ox properties as well as structural diversity. In this study, the antioxidant activity of *Ziziphus lotus* was assessed using DPPH assay (Figure 2). Due to ease of reaction, the DPPH radical method is extensively used to evaluate free radical scavenging activity and the concentration of a species to scavenge 50% DPPH radical (EC50) was mainly determined to get better estimation on sample antioxidant efficiency (Table 2). In DPPH assay, the EC50 value was found to be 131.01 mg/mL in methanolic extract. Furthermore, the BHT values are higher than those of the methanolic extract. Nevertheless, with greater concentrations, the free radical scavenging activity of *Ziziphus lotus* fruit is closely similar to BHT, as the inhibition percentage of the extract is near the values of BHT.

In addition, radical inhibition activity of *Ziziphus lotus* extract was determined using ABTS radical decolorization assay (Figure 3). Here, the percentage of the ABTS radical inhibition activity reached a peak of 95.85% at a concentration of 200 mg/mL. We found that the ABTS values of the methanolic extract are higher than those of Trolox leading to the conclusion that *Ziziphus lotus* fruit has good antioxidant properties (Figure 3). It has been reported that Ziziphus jujube had good antioxidant activity against DPPH as well as a straight correlation with phenols content [40]. Furthermore, the potential antioxidant of *Ziziphus lotus* could be also related with the content and type of metabolites present in its extract [41,42]. Alkaloids are amongst the well-known metabolites for their antioxidant activity; they are spread in the entire parts of the plant. For instance, betulinic acids, which are natural pentacyclic triterpenoids extensively disseminated in all parts of the plant, showed high antioxidant activity [43]. However, further phytochemical study focusing on the isolation and characterization of the constituents in *Ziziphus lotus* extract liable to its bioactivity is still required.

Conclusion

Our study showed that *Ziziphus lotus* fruit can be a source of plant antioxidants with the possibility of usefulness in foodomics, cosmetics and

pharmaceutical fields. The phenolic compounds might be the major active substances responsible for the highest antioxidant activity. Furthermore, the richness of *Ziziphus lotus* fruit and its potent antioxidant effect may explain its efficiency against bacteria. This could be also explained by the usefulness of this plant in treating several infections and health issues which is interesting for designing an antibacterial agent from vegetable source.

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Table 1. Antibacterial properties by disc difusion method (inhibition zone diameter in mm) of *Ziziphus lotus* extracts as well as its methanol extract antibacterial activity by micro dilution method on different bacteria.

Test agents	Bacterial species				
	E.Coli	Agrobacterium sp	Rhizobium sp	Bacillus pumilus	Bacillus subtilis
A (94.28 mg/mL)	-	-	-	-	-
B (3.03 mg/mL)	11	11	16	12	7
MIC (µg/ml) in B	400	400	3.2	320	340

A: water extract; B: methanol extract

Table 2. DPPH's and ABTS's IC_{50} as well as EA values of the *Ziziphus lotus* extract.

	IC_{50} (µg/ml)	**EA**
DPPH		
Methanolic extract	131.01	0.00763
BHT	50.67	0.0197
ABTS		
Methanolic extract	52.4207	0.0191
Trolox	63.44	0.0158

Figure 1. Phenolic and flavonoid content of *Ziziphus lotus* in methanolic extract.

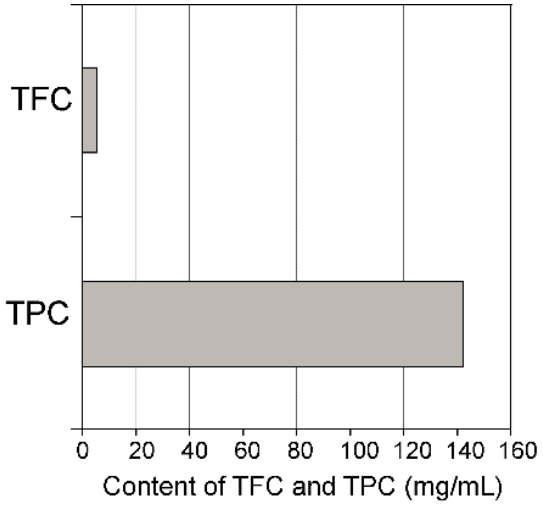

Figure 2. Free radical scavenging activity of *Ziziphus lotus* in methanolic extract.

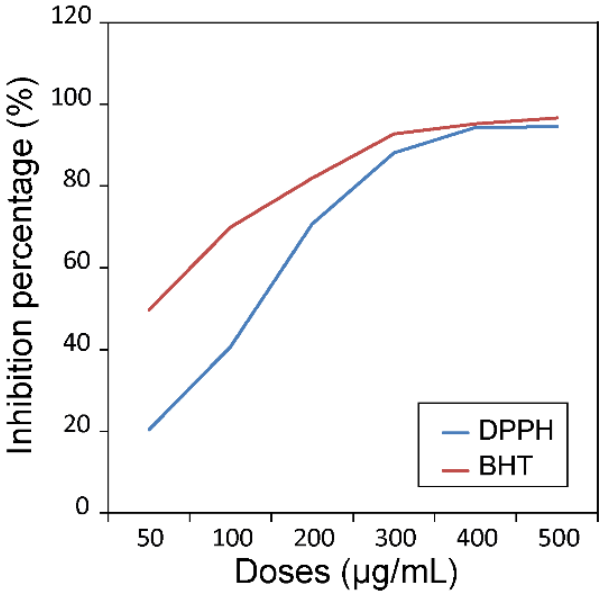

Figure 3. ABTS radical scavenging activity of *Ziziphus lotus* in methanolic extract with different concentrations.