Antimicrobial and radical scavenging activities of Moroccan Ziziphus lotus L. seeds

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ABSTRACT

Natural plant extracts contain a variety of phenolic contents, which are associated to various biological activities. In this study, we evaluated the antioxidant and antimicrobial activities of organic (ethanol and methanol) and aqueous extracts prepared from Ziziphus lotus L. seeds. The total polyphenol content of the extracts was determined using the Folin-Ciocalteu reagent, it was in the range of 50.67 ± 1.44 (Ethanolic Extract), 39.32 ± 1.44 (Methanolic Extract) and 23.54 ± 0.44 (Aqueous Extract) mg gallic acid equivalent/g DW. The content in flavonoids was estimated at 69.19 ± 0.10 (Ethanolic Extract), 53.13 ± 0.55 (Methanolic Extract) and 9.63 ± 0.88 (Aqueous Extract) mg equivalent quercitin/g DW. The condensed tannin assay revealed that the methanol extract was rich on tannin (9.12 ± 1.07 mg/g) relatively to the ethanol and aqueous extracts (4.97 ± 0.95 and 1.88 ± 0.47 mg/g respectively). The antioxidant activity was evaluated in vitro by DPPH and phosphomolybdate (total antioxidant capacity). The results reveal that the three extracts have a capacity to trap the DPPH radical with IC50 values ranging from 1.33 ± 0.01 to 3.11 ± 0.05 mg/ml for the methanol, ethanol and aqueous extract respectively. This antioxidant activity is confirmed by the phosphomolybdate test. The antimicrobial activity of the studied extracts was evaluated using the broth microdilution technique, on five microbial strains: Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis and Candida tropicalis. Results revealed an activity on the four bacterial strains tested. While the fungal strain (Candida tropicalis) showed an amount of resistance to the tested extracts. All this results could justify the use of Ziziphus lotus in the treatment of various infections in traditional environments.

Keywords: Ziziphus lotus, Phenolic compounds, Antioxidant activity, Antimicrobial activity.

INTRODUCTION

The jujube (Ziziphus lotus L.), commonly known in Morocco as "Sedra", is an aromatic and medicinal plant widely used in traditional medicine to treat diarrhea, ulcers, fevers, and used as a sedative [1]. In addition, Ziziphus lotus fruits are described as emollients and included in the treatment of throat and bronchial irritation lungs [2, 3]. The jujube is abundantly present in North Africa ( Libya, Algeria and Morocco) and in southern Europe especially Spain, Sicily, Greece and Cyprus [4]. Several studies have suggested that the phytochemical content and anti-oxidant effect of plants contribute to show protective effect against chronic and degenerative diseases [5, 6]. Recently, there has been a renewed interest in the therapeutic potential of medicinal plants as antioxidants in the reduction of these free radical-induced tissue lesions [7]. In fact, free radicals play a role in the pathogenesis of chronic degenerative diseases, including cancer, autoimmune diseases, inflammatory diseases, cardiovascular diseases, neurodegenerative diseases and aging in general [8, 9, 5]. Because of these facts, synthetic and natural compounds with potential antioxidant activity received an increased attention in biological research, medicine and pharmacy [5, 6].

Multidrug-resistant microorganisms are difficult to treat [10]. Common multidrug-resistant pathogens, such as Staphylococcus aureus and Pseudomonas aeruginosa, demonstrate a high resistance to the majority of drugs [11]. The development of MDR (Multi Drugs resistant) bacteria has led many authors to invest in the research for new natural molecules with antimicrobial activity. These sought molecules must possess various chemical properties and useful against these pathogenic microbes [12].

Current research on the different pharmacological activities of Ziziphus lotus L. and its derivatives are of great importance for modern medicine. However, this species is often ignored or even forgotten. It is becoming increasingly degraded because of the impact of ever-increasing human factors (overgrazing, grubbing up by farmers to change the purpose of the land, etc.).
This looting is likely to jeopardize the survival of the species. Due to lack of pharmacological uses information of *Z. lotus* seeds, and in the aim of enhancing the potential that culminate this plant, we conducted this study to highlight its antimicrobial and antioxidant activities. Thus, the objective of this study is to evaluate the antioxidant, antimicrobial activities and determine the concentration of some secondary metabolites of *Z. lotus* seed extracts.

**MATERIAL AND METHODS**

**Plant material**

Plant used consists of *Ziziphus lotus* seeds, collected in August from Fez (Zouagha-Moulay Yaâcoub) in Morocco. The climatic and geolocalisation data are summarized in table 1.

**Preparation of extracts**

Seeds extracts were obtained using three solvents (methanol, ethanol and distilled water). The aqueous extract was prepared by Soxhlet apparatus according to plant: solvent ratio of 1/10 (w/v) for 4 hours.

The seeds were crushed with a mortar and sieved to obtain a fine and homogeneous powder; which will be used for extraction.

**Phytochemical screening of the plant extract**

**Total polyphenols contents**

The total phenolic contents were performed according to the method of Singleton and Rossi (1985) \(^{13}\) which consists of mixing 1 ml of extract with 0.5 ml of Folin-Ciocalteu reagent (1:1 distilled water) and 2.5ml of sodium carbonate solution (20%). The mixture was vortexed, then incubated for 2 h in the obscurity. The absorbance was determined at 725 nm by an UV-visible spectrophotometer The calibration curve was performed with gallic acid and the final concentrations were expressed in mg of gallic acid equivalent (GAE)/mg of extract.

**Total flavonoids**

Total flavonoids content was performed according to the aluminum trichloride’s method described by \(^{14}\). Indeed, 0.3 ml of NaNO\(_2\) 5% was mixed with 1 ml of extract. After 5 min, 0.3 ml of AlCl\(_3\) 10% was added. Once 6 minutes have elapsed, 2 ml of NaOH 1M were added and the total volume was made up to 10 ml with distilled water. The absorbance was measured at 510 nm. Total flavonoids content was calculated from the calibration curve established with quercetin standard, and were expressed as mg of quercetin equivalent (mg QE)/g of extract.

**Determination of condensed tannins**

Condensed tannins were performed according to Ribereau-Gayon and Stonestreet (1966) \(^{15}\). Briefly, 1 ml of extract diluted 2 times were mixed with 1.5 ml of HCl and 0.5 ml of distilled water. The mixture was heated for 30 min in a water bath at 100°C. The optical density was measured at 550 nm. The difference in optical density between the hydrolysed tube and the control (tube at room temperature) corresponds to the amount of tannins present in the extract. The results are expressed by following relation:

\[
\text{Tannins (mg/ml)} = (\text{OD}_{\text{hydrolysis}} – \text{OD}_{\text{Control}}) \times 19.33
\]

**Antimicrobial assays**

From a 24 h-old pure agar culture, 1 to 2 colonies were transferred in sterile saline for each strain. Then, the solutions were well-vortexed and their turbidity were adjusted to 0.5 McFarland.

The minimal inhibitory concentration (MIC), is defined as the lowest concentration of the extract that inhibits the microbial growth. For its determination, the broth microdilution was performed according to \(^{16}\). The microplates were incubated for bacteria at 37 °C for 24 h and at 30 °C for 48 h for *C. tropicalis*. After incubation, 10 μl of resazurine dye are added to reveal the microbial growth.

To determine the MBC/MIC, 3 μl of the negative wells were deposited on the surface of LB/YPG agar plate and incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for *C. tropicalis*. After incubation, the MBC or MFC has been recorded as the lowest concentration yielding a negative visible growth. To highlight the nature of the antimicrobial effect of the extracts tested the MBC/MIC ratio was calculated (Levison, 2004). \(^{17}\)

**Antioxidant activity**

**DPPH radical scavenging assay**

The free radical DPPH was used to evaluate the antioxidant capacity of the extracts. For the extracts, a serial dilution was prepared from a stock solution of 4 mg/ml prepared in methanol. Then, 1 ml of each dilution was added to 1 ml of DPPH (0.004 %). The mixture was left in the dark for 30 minutes, and the discoloration against the negative control containing only the DPPH solution was measured at 517 nm. The percentage of antioxidant activity was calculated according to the following equation:

\[
\text{Antioxidant activity (\%)} = \frac{\text{Abs DPPH} – \text{Abs final}}{\text{Abs DPPH}} \times 100
\]

The antioxidant capacity of the various extracts studied was interpreted from their IC\(_{50}\), that scavenges 50 % of the initial DPPH \(^{18}\). The value of IC\(_{50}\) is expressed in μg/ml. The results obtained are expressed in μg equivalent BHT/ ml.

**Total antioxidant capacity**

The phosphomolybdenum method was also used to evaluate the antioxidant activity of the extracts \(^{19}\). Indeed, 200 μl of the extract


| Climate Data | Station |
|--------------|---------|
| Latitude     | FEZ     |
| Longitude    | 34°02′13 N |
| Altitude     | 4°59′59 W |
| Rainfall mm/year | 375 |
| Bioclimatic stage | Semi-Arid |

N: North; W: west

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were mixed with 3 ml of reagent solution (sulfuric acid, sodium phosphate and ammonium molybdate). The screwed tubes were incubated at 95 °C for 90 minutes. Then, the absorbance was measured at 695 nm. The total antioxidant capacity is expressed in milligram equivalent of vitamin C (mg Evitamin C), from a calibration curve, carried out by vitamin C standard.

Method of statistical analysis

The data obtained were the subject of a statistical analysis (averaging, analysis of variance ANOVA, SE) to search the variability between the different factors studied. A post-hoc test of Tukey was made whenever there was a significant effect of factor studied by ANOVA.

RESULTS AND DISCUSSION

Yield of extracts

The yield of extracts is presented in Fig 1. The highest yield is observed at methanol extract (13.1%) followed by aqueous (9%) and ethanol extracts (6.5%). The ANOVA showed a highly significant difference between the three extracts studied (ANOVA: \( F = 49.562; \text{ddl} = 2; P \leq 0.001 \)).

This results are in disagreement with Djemai [20], which revealed that the yield of the aqueous extract of Z. lotus fruit is higher (40.4%) than methanol extract (6.3%). This could be explained by the variation of chemical constituents of the seeds and fruits. Bourgou et al. [21] researched on the effect of the solvent and the extraction method on the phenolic content and the antioxidant potential of Euphorbia helioscopia. They showed that the solvent has a significant influence on the yield. It can be noted that temperature could play a positive effect by improving the extraction of the compounds for aqueous extract, this justifies the choice of the Soxhlet apparatus [22]. Pelagie et al. [23] conducted a comparative study of the phenolic contents and the antioxidant activities of Garcinia kola (Guttiferaeae) and Cucumerspis edulis (Cucurbitaceae) seeds. According to their results, the aqueous extract of Cucumerspis edulis has a higher yield (14%) compared to the ethanol extract (10%). However, for Garcinia kola seeds, the yield was higher in the ethanol extract (30%) than in the aqueous extract (8.8%).

Determination of secondary metabolites in the extracts

Phenols content

The phenolic content of methanol, ethanol and aqueous extracts is shown in fig 2. Results showed that the ethanol extract had a high total phenols content (50.67 ± 1.44 mg/g). On the other hand, the aqueous extract has the lowest concentration (23.54 ± 0.44 mg/g). The ANOVA showed a highly significant differences between the three extracts (ANOVA \( F = 134.498; \text{ddl} = 2; P \leq 0.001 \)).

This Results disagrees with Khouchlaa et al. [24], who show that the highest phenols content of Z. lotus fruits was recorded in aqueous extract. In addition, a study carried out on the aqueous extracts of Ziziphus mauritiana and Eriobotrya japonica fruits, revealed that the phenols content of these extracts was largely low compared to our aqueous extract [25]. Several factors may influence the phenolic contents, such as extrinsic factors (geographical and climatic factors), genetic factors, and the degree of maturation and the duration of storage [26, 27].

Total flavonoids content

The total flavonoids content for each extract is shown in Fig 3. The results revealed that the ethanol extract had a high content of flavonoids (27.68 ± 0.04 mg EQ/g), followed by the methanol extract (21.25 ± 0.20 mg EQ/g) and the aqueous extract with a low concentration (3.85 ± 0.35 mg EQ/g). The ANOVA showed a highly significant difference for all studied extracts (ANOVA \( F = 8,240; \text{ddl} = 2; P \leq 0.001 \)).

Content of condensed tannins

The analysis of the results showed a high condensed tannin content of the methanol extract (9.12 ± 1.07 mg/g) compared to the other two extracts studied (Fig 4). The ANOVA showed a highly significant differences between the condensed tannin contents of the three extracts (ANOVA \( F = 17.61; \text{ddl} = 2; P \leq 0.001 \)).
Some studies on the phytochemical and biological activity of *Ziziphus lotus* L. fruit showed that the aqueous extract is richer in tannins (6.77 ± 1.95 μg ECT/mg of extract) followed by the methanol extract (4.57 ± 0.94 μg ECT/mg of extract) [20].

### Antimicrobial activity

#### Minimal inhibitory concentration

The MIC values of extracts against target strains are given in Table 2. Ethanol extract showed a low MIC about 50 mg/ml against *E. coli*, *P. aeruginosa* and *E. faecalis*. For methanolic extract, the concentration corresponding to 50 mg/ml inhibited growth of *P. aeruginosa* and *E. faecalis*. Similarly, aqueous extract had an antimicrobial activity against the different strains studied and the low MIC obtained was 100 mg/ml detected against *P. aeruginosa* and *E. faecalis*. It can be concluded that the ethanol, methanol and aqueous extracts had an antibacterial effect against the four bacterial strains tested. *E coli* showed high sensitivity to ethanolic extract, whereas *Candida tropicalis* was more resistant.

![Figure 4: Content of condensed tannins of different types of *Z. lotus* seed extract. Vertical bars correspond to SE for n = 3](image)

| Table 2: MIC of *Z. lotus* seed extracts on the tested microbial strains. |
|--------------------------|---|---|---|---|---|---|---|---|---|---|---|
|                         | Ethanol Extract (EE) |
|                         | [C] (mg/ml) | 200 | 100 | 50 | 25 | 12.5 | 6.25 | 3.12 | 1.56 | 0.39 | 0.19 | T    |
| *E. coli*               | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *P. aeruginosa*         | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *S. aureus*             | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *E. faecalis*           | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *C. tropicalis*         | +    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
|                         | Methanolic Extract (ME) |
|                         | [C] (mg/ml) | 200 | 100 | 50 | 25 | 12.5 | 6.25 | 3.12 | 1.56 | 0.39 | 0.19 | T    |
| *E. coli*               | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *P. aeruginosa*         | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *S. aureus*             | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *E. faecalis*           | -    | -   | ○   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *C. tropicalis*         | +    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
|                         | Aqueous Extract (AE) |
|                         | [C] (mg/ml) | 200 | 100 | 50 | 25 | 12.5 | 6.25 | 3.12 | 1.56 | 0.39 | 0.19 | T    |
| *E. coli*               | ○    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *P. aeruginosa*         | ○    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *S. aureus*             | ○    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *E. faecalis*           | ○    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |
| *C. tropicalis*         | +    | +   | +   | +  | +  | +   | +    | +    | +    | +    | +    | +    |

The bioactive molecules of Ethanolic Extract of *Ziziphus lotus* appear to have the strongest inhibitory effect against most studied strains. Our results are consistent with Ertürk [28] who found that the ethanol extract of cumin is more active against *Escherichia coli* and *Pseudomonas aeruginosa*, compared with methanol extract. Similarly, the results of Mohsen and Anmar [29] proved that ethanol was the best solvent for the extraction of phenolic compounds, followed by methanol and finally water. This could explain the richness of ethanolic extract in phenols contents, and its effectiveness against most studied strains compared to other extracts.

It appears that *Enterococcus faecalis* (Gram+) was the most susceptible bacteria in comparison with the other strains (Gram-), this can be attributed to the difference in the structure of the cell wall between Gram+ and Gram- bacteria. The cell walls of Gram+ bacteria consist of a single layer while the Gram- cell wall has a multilayer structure.
bound by an outer cell membrane [30]. This makes Gram- bacteria more resistant. The results of phenols contents, flavonoids and condensed tannins revealed that EE was rich in these metabolites compared to methanolic extract and aqueous extract. This could explain its antimicrobial activity.

**Minimal bactericidal concentration**

The MBC values of the three studied extracts are showed in Table 3. In fact, the most interesting MBC was 100 mg/ml, shown against P. aeruginosa, E. faecalis and E. coli by Ethanol Extract. For the methanolic extract, the MBC of S. aureus and P. aeruginosa were 200 mg/ml, whereas the MBC of E. Coli and E. faecalis were greater than 200 mg/ml. AE had a MBC of 100 mg/ml against E. faecalis and 200 mg/ml against P. aeruginosa, E. coli and S. aureus. According to MBC/MIC ratio, it is appeared that the extracts of the Ziziphus lotus seeds had a bactericidal activity on the studied strains.

**Table 3: Antibacterial parameters of extracts and their interpretation**

| Extract Type | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC | Interpretation |
|--------------|-------------|-------------|---------|----------------|
| Ethanolic (EE) | E. coli: 50  | 100  | 2 | Bactericidal |
| | S.A: 100  | 200  | 2 | Bactericidal |
| | P.A: 50  | 100  | 2 | Bactericidal |
| | E.F: 50  | 100  | 2 | Bactericidal |
| Méthanolic (ME) | E. coli: 100  | >200  | -- | -- |
| | S.A: 100  | 200  | 2 | Bactericidal |
| | P.A: 50  | 200  | 4 | Bactericidal |
| | E.F: 50  | >200  | -- | -- |
| Aqueous (AE) | E. coli: 200  | 200  | 1 | Bactericidal |
| | S.A: 200  | 200  | 1 | Bactericidal |
| | P.A: 100  | 200  | 2 | Bactericidal |
| | E.F: 100  | 100  | 1 | Bactericidal |

**Antioxidant activity**

**DPPH radical scavenging assay**

The antioxidant activity of the methanol, ethanol and aqueous extracts of Ziziphus lotus seeds is showed in Fig 5. It can be noted that the percentage of inhibition for the extracts was lower than the standard. At a concentration of 4 mg/ml, the highest percentage of DPPH inhibition was detected in the methanolic extract (90.12% ± 1.73), while the aqueous extract has the lowest concentration (54.29% ± 1.13). The IC50 detected for BHT (0.24 ± 0.001 mg/ml) was superior in comparison with the different extracts studied. The ethanol and methanol extracts respectively had an IC50 in the order of 1.33 ± 0.008 and 1.32 ± 0.09 mg/ml. On the other hand, the aqueous extract showed a higher concentration (1.19 ± 0.01 mg/ml). This allowed us to emphasize that the ethanol extract represents the most active extract.

**Figure 5:** inhibition percentage of the DPPH radical of the reference antioxidant and the three extracts tested. MS: Stock solution: 4mg / ml; D1: 2mg / ml; D2: 1mg / ml; D3: 0.5 mg / ml; D4: 0.25mg / ml. Vertical bars correspond to SE for n = 3.

The ANOVA showed a highly significant difference between the ethanol, methanol and aqueous extracts (ANOVA: F = 1907.163; dd1 = 2; P≤0.001).

Rajinder et al. [25] tested the antiradical activity of the aqueous extract of the Ziziphus mauritiana fruit and Eriobotryajaponica with a concentration of 200 mg/ml. The inhibition percentage for Z. mauritiana was 74.89% and 51.07% for Eriobotryaja ponica. This activity appears weak compared to our aqueous extract, which showed a strong inhibition (54.29%) at 4 mg/ml. The strong activity of our methanol extract could be due to the presence of phenolic compounds in high concentration, especially the condensed tannins (9.12 mg/g). Similarly, Bougandoura and Bendimerad [31] showed that antioxidant molecules such as ascorbic acid, tocopherol, flavonoids and tannins, reduce and discolor the DPPH due to their ability to liberate hydrogen. On the other hand, it is well established that the antioxidant activity is positively correlated with the structure of the phenols. Generally, phenols contents with a high number of hydroxyl groups have the highest antioxidant activity [35], due to their ability to give more atoms to stabilize the free radicals [33]. Thus, the antioxidant activity is not only dose-dependent but also structure-dependent [34].

**Table 4: Antioxidant potency (expressed as IC50 in mg/ml) of the reference antioxidant**

| Extract Type | IC50 ±Ecart type (mg/ml) |
|--------------|--------------------------|
| BHT          | 0.24±0.001               |
| Ethanol Extract | 1.33±0.008             |
| Methanol Extract | 1.32±0.09          |
| Aqueous Extract | 3.11±0.05             |

**Total antioxidant capacity**

The phosphomolybdate test of the various extracts was presented in Fig 6. The estimation of the total antioxidant capacity showed a variability as a function of the nature of solvent. (ethanol, methanol and aqueous). It can be noted that for the concentration 4 mg/ml, the methanol extract has a strong total antioxidant capacity, followed by the ethanol and aqueous extracts. This result confirms the DPPH test. The ANOVA showed a highly significant differences between ethanol, methanol and aqueous extracts (ANOVA: F = 1907.163; dd1 = 2; P≤0.001).
CONCLUSION

The results show that the ethanol extract is the richest in phenolic contents compared to the other studied extracts. The evaluation of the antimicrobial activity showed a bactericidal activity of the ethanol, methanol and aqueous extracts of the Ziziphus lotus seeds against the microbial strains tested, with resistance of Candida tropicalis, MIC and MBC vary according to the extraction solvent. In addition, the methanol extract showed a very important antioxidant activity. It can be indicated that the seeds of Z. lotus appear an antioxidant reservoir that can be used in the fight against free radicals.

Moreover, it seems of great importance to extend the range of antimicrobial tests, as well as the isolation and characterization of the active compounds in the various extracts, in order to identify the elements responsible for the biological activities of this plant.

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