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Quantification of total phenols, flavonoides and tannins from Ziziphus jujuba (mill.) and Ziziphus lotus (l.) (Desf). Leaf extracts and their effects on antioxidant and antibacterial activities

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Abstract: This work was performed to determine the biochemical composition, antioxidant and antimicrobial activities of leaf extracts collected from four different provenances: Mahdia and Mahres (*Ziziphus jujuba*); Kairouan and Rouhia (*Ziziphus lotus*). Total phenols, flavonoids, tannins contents and antioxidant activity were evaluated using the Folin ciocalteux, Aluminum trichloride, vanillin and scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals methods, respectively. The antimicrobial activity was evaluated against three bacterial strains (*Escherichia coli*, *Staphylococcus aureus* and *klebsiella pneumoniea*) and three fungal strains (*Fusarium culmorum*, *Fusarium solani* and *Botrytis cinerea*), according to well Agar diffusion method. Total phenols and flavonoids were present at levels of 21.98 mg GAE /g DW and 7.80 mg ER/g DW; respectively in *Ziziphus lotus*. These levels did not exceeded 13.70 mg GAE /g DW and 6.73 mg ER/g DW for *Ziziphus jujuba*. The tannin contents were present in equal levels (7.9 mg EC/g DW) in two species. The high antioxidant activity (0.01 µg/ml) was noted in Rouhia provenance. The *Ziziphus lotus* leaf extracts showed promising efficiency against all tested microorganisms with a zone of inhibition ranging between 22 and 23.5 mm. This study could validate the medicinal potential of *Ziziphus* specie and explain why tunisian people traditionally use it in medicine to treat several pathologies. *Ziziphus* leaf extracts may be suggested in foods and pharmaceutical industries. Leaf extracts proved also to be effective against tested microorganisms. So, an adequate toxicological study must be carried out to verify the possibility of using these plants for fighting microorganisms.

Keywords: *Ziziphus*, phenols, condensed tannins, antioxidant activity, antimicrobial activity

1. Introduction

Nowadays, there is an upsurge of interest on medicinal plants that have attracted more and more attention due to their therapeutic properties and pharmacological activity [1]. Polyphenols were one class of secondary metabolites that were used in plant adaptation against unfavorable conditions (salinity drought, temperature…), but also in organism prevention

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against some illness such as cancer, inflammatory and cardiovascular disease [2]. Flavonoids, known as vitamin P, were mostly used in plants to produce yellow and other pigments, which play an important role in the colors of plants.

The jujube species, a medicinal tree menaced, were present, in Tunisia, in three species: *Ziziphus lotus* (l.), *Ziziphus jujuba* (mill.) and *Ziziphus spina-christi* (l.). *Z. jujuba* was widely studied for its therapeutic and alimentary uses [2-4]. In fact, according to these authors, tunisian *Z. jujuba* extracts (leaves, seeds or pulps) were rich on fatty acids (linolenic, palmitic, oleic, linoleic acids), sterols (sitosterol, stigmasterol…) and flavonoïds (rutin and apigenin). This richness improved the use of these organs in cosmetics and in pharmacology. *Zizyphus spina-christi* and *Ziziphus lotus* had been used in folk medicine as a demulcent, depurative, anodyne, emollient, stomachic, These characteristics make those species with valuable multi-purpose shrubs for semi-arid to arid ecological areas [2, 5].

In order to validate the medicinal potential of this specie and to explain why tunisian people traditionally use it to treat several pathologies, this study was carried out to evaluate the phytochemical constituents and antioxidant potential of the leaf extracts of four *Ziziphus* provenances: Mahres and Kairouan (*Z. lotus*), Rouhia and Mahdia (Z. *jujuba*).

2. MATERIAL AND METHODS

2.1. Plant material

Leaves and were sampled in summer 2014. The identification of the plant material was done by Professor Mohamed Boussaid and a voucher specimen of the plant was deposited at the Herbarium of INRGREF (Tunisia). The leaves were dried at room temperature during a half month in a dry and airy environment. Dried leaves were grounded by a mill equipped with a grid whose holes were 1.00 mm in diameter and stocked in plastic bags in the dark until chemical analysis. Leaf powders (1 g) were submitted to maceration with 10 ml of pure methanol for 30 min. The extracts, filtered through Whatman N°1 filter paper, were pooled and concentrated under vacuum.

2.2. Chemical Reagents

Folin-ciocalteu, phenol, DPPH, gallic acid, catechin sodium carbonates, hydrochloric acid and methanol were purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents used were of the highest purity.

2.3. Total phenol and flavonoid contents

The total phenolic content was estimated flowing the Folin-Ciocalteu method as described by Singleton et al., (1965) with slight modifications. From each sample, 0.5 ml of methanolic solution was added to 2.5 ml of Folin- Ciocalteu reagent and 2 ml of sodium carbonate (75 g/l) solution. The reading of the absorbance was done at 765 nm using a Shimadzu 1600-UV spectrophotometer after incubation during 30 min. The same procedure was repeated for gallic acid used as standard. Total phenols of each fraction, expressed into mg GAE/g DW, were measured using the regression equation of a calibration curve $y = 0.0114x + 0.518$, $R^2 = 0.9932$.

The total flavonoid contents were assayed using the Aluminum trichloride method (Earp et al. 1981). 1 ml of $AlCl_3$ was added to 1 ml of plant extract. The volume was adjusted to 25 ml with methanol and thoroughly mixed. The absorption was measured after 40 min by a Shimadzu UV-1600 (Tokyo, Japon) spectrophotometer at 420 nm. Flavonoid contents were expressed as mg quercetin equivalent /g DW. The calibration curve range was 0-50 µg/mL ($R^2 = 0.981$). All samples were analyzed in three replications.

2.4. Condensed tannin contents

Condensed tannins were determined by colorimetric analysis [7]. A mixture of vanillin (1 ml) and 4 ml of HCl were added to 200 µl of leaf extracts and incubated 20 min in obscurity.

Catechin was used as a standard (0- 1250 µg/ml). All measurements were performed in triplicate. After agitation, absorbance's were read at 500 nm using a Jenway 6100 spectrophotometer. The results were expressed as microgram catechin equivalent per gram dry weight (µg CE/g DW)

2.5. Antioxidant activity

The working solution was prepared by mixing the two stock solutions in equal proportions and allowing them to react for 12-16 hours. This solution was stored in a dark place at room temperature. Before use, the solution was diluted with ethanol to obtain absorbance between 0.800 and 1.000 nm. This solution was mixed with sample (2.5 to 50 µg mL^{-1}) or standard solutions. A control containing methanol and DDPH solution was also realized. The absorbance was read at 734 nm after 30 min of incubation at 25°C.

In test tubes, 2.36 mg of DPPH previously dissolved in 100 ml of ethanol, mixed and incubated in obscurity. The leaf extracts were diluted with methanol (0.75 to 0.125 µg/ml) before use. The absorbance was read at 490 nm after 30 min incubation in dark place. Measurements for each experiment were done in triplicate. Antioxidant activity, expressed as inhibitory effect of the DPPH radical, was calculated using this formula:

$$\text{The percentage of inhibition} = [(A_0 - A_c) / A_0] \times 100$$

where A_0 was the absorbance of the control and A_c was the absorbance of the plant extract/ standard. The IC50 value, the concentration (in µg/ml) of the compound required to scavenge DPPH radical by 50, was determined graphically by the linear regression [8].

2.6. Antimicrobial activity

The test microorganisms used in this study were: *Escherichia coli* (ATCC10536), *Staphylococcus aureus* (ATCC 6538) and *klebsiella pneumoniea* (ATCC 10031). These species were generously provided by Laboratory of Ecology, Technology and Microbiology (INSAT). The *Fusarium culmorum*, *Fusarium solani* and *Botrytis cinerea* were obtained from the culture collection of the Tunisian National Institute of Agronomic Research (INRAT). The bacterial and fungal stock cultures adjusted to suspension of 10^6 cells were incubated for 24 hours at 37°C on potato dextrose agar (PDA) medium and were refrigerated at 4°C. Antibacterial tests were evaluated using well Agar diffusion method under strict aseptic conditions. So 100µl of suspension had been placed in 5 ml of melted cooled test agar [9, 10]. It was inoculated by flooding on Petri dishes containing agar culture medium BTCS. After agar solidification, three wells (10 mm in diameter) were bored using a sterile cork borer. Three concentrations of each leaf extracts (5; 60 and 100 mg/mL) were prepared and dripped directly into the first, the 2nd and 3rd well, respectively with micropipette. Sterilized distilled water was used as a negative control which was introduced into the 4th well. After 24h of incubation at 37°C, the diameter of inhibition zone surrounding each well was measured.

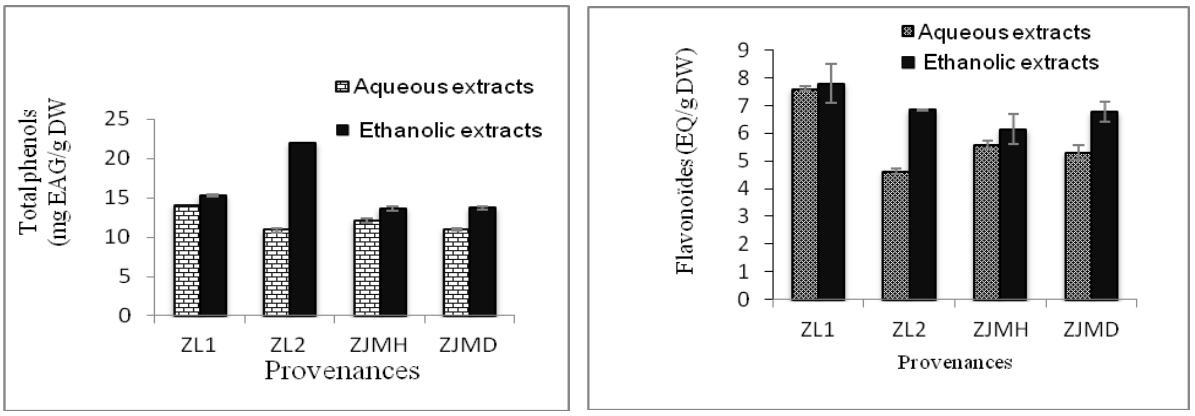
2.7. Statistical analysis

Results were statistically evaluated using STATISTICA. Data from three samples was reported as means ± standard deviation. Differences were tested with the ANOVA procedure using the Duncan test with a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Total phenol and flavonoid contents

The results indicated that 1 g DW of *Ziziphus* leaves contained between 13.62 and 21.98 mg GAE of phenolic contents. The $EEZL_2$ was the richest one. The methanol was found to be the efficient solvent to extract *Ziziphus* leaves. This idea was also confirmed by Medini et al. (2014). The relative amounts of phenols in *Z. jujuba* leaves found in this study were greater more important than those done (6.04 mg EAG/g DW) by Elaloui et al. (2016). This variability could be explained by many factors including the origin, the period of harvest, the age and the stage of plant development. Other environmental factors (temperature, altitude, sunshine, animal aggression and diseases) could also influenced this variability. The physiological stage could also the polyphenol contents and biological activity [12].



a **b**

Figure 1. Total phenolic (a) and flavonoid (b) contents of four provenances of *Ziziphus* leaf extracts, **ZL1:** *Z. lotus* (Kairouan), **ZL2:** *Z. lotus* (Rouhia), **ZJMH:** *Z. jujuba* (Mahres), **ZJMD:** *Z. jujuba* (Mahdia).

1. The data are the mean values of three measurements ± SD (standard deviation)
2. The confidence intervals were calculated at the threshold of 5%.

As noted in figure, the total flavonoids contents varied between study sites and provenances of collection. It ranged between 5.30 - 6.73 and 4.63 - 7.80 for *Z. Lotus* and *Z. jujuba* respectively. It has been proved that the levels of total phenols and flavonoids were high when the environment conditions of the plant were not adequate. In this case, the plant promoted the synthesis of secondary metabolites in order to adapt and survive [13].

3.2. Condensed tannin levels

Results proved that condensed tannin levels oscilled between 4.38 and 6 mg EC/g DW for aqueous extracts (figure 2). Level were higher than ethanolic extracts that attempt levels between 7.05 and 8 mg EC/g DW.

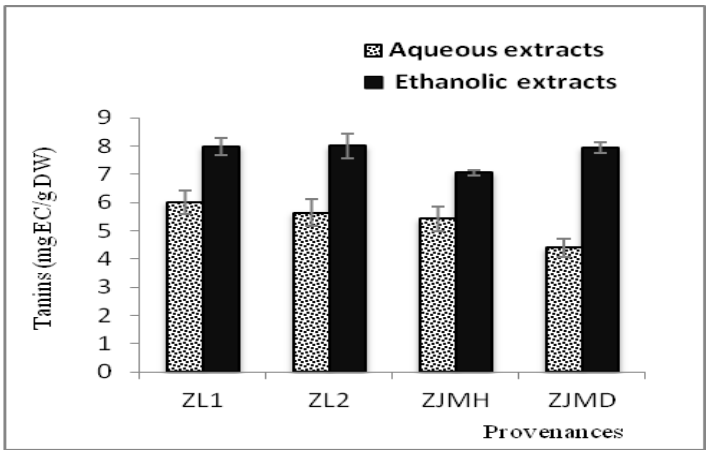


Figure 2. Condensed tannin contents of four provenances of *Ziziphus* leaf extracts, **ZL1:** *Z. lotus* (Kairouan), **ZL2:** *Z. lotus* (Rouhia), **ZJMH:** *Z. jujuba* (Mahres), **ZJMD:** *Z. jujuba* (Mahdia).

1. The data are the mean values of three measurements ± SD (standard deviation)
2. The confidence intervals were calculated at the threshold of 5%.

As shown in this figure, the effect of solvent in tannin solubility showed the same classification as phenolic and flavonoid contents.

3.3. Antioxidant activity

The leaf of *Z. lotus* of Rouhia provenance registered the highest activity (0.01 mg/ml). The EAZJMD showed high antioxidant activity (CI_{50} =0.61 mg/ml).

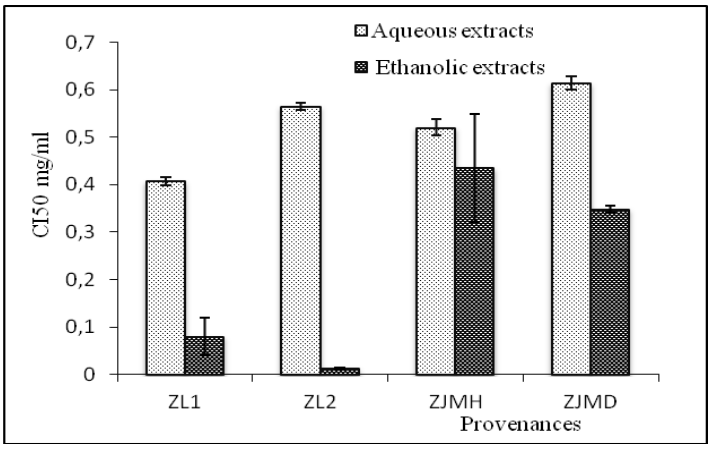


Figure 3. Inhibitrice concentration of four provenances of *Ziziphus* leaf extracts, **ZL1:** *Z. lotus* (Kairouan), **ZL2:** *Z. lotus* (Rouhia), **ZJMH:** *Z. jujuba* (Mahres), **ZJMD:** *Z. jujuba* (Mahdia

1. The data are the mean values of three measurements ± SD (standard deviation)
2. The confidence intervals were calculated at the threshold of 5%.

In fact, a linear correlation ($R^2 = 0.795$) had been observed between the CI_{50} of Z. leaf extracts and their phenolic levels (Figure 4). In fact, phenolic levels and antioxidant activity varied in the same way. Our results corroborate by Al-Jassabi and Abdullah et al. (2013). The antioxidant capacity levels were maximum at the flowering stage [11].

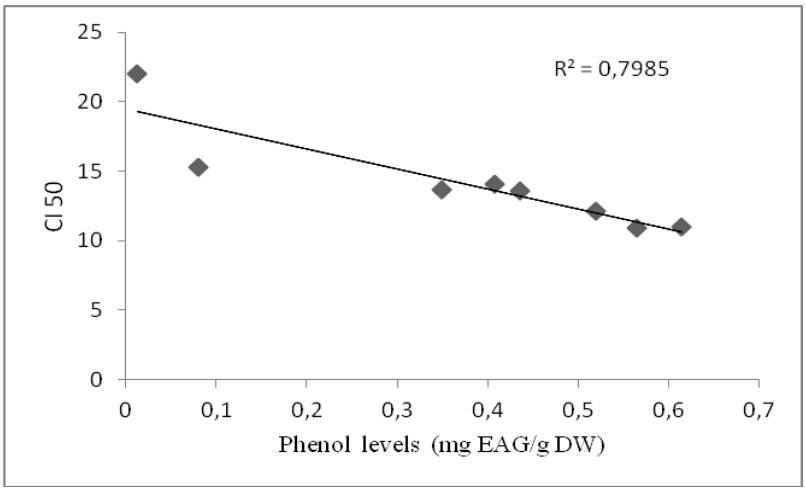
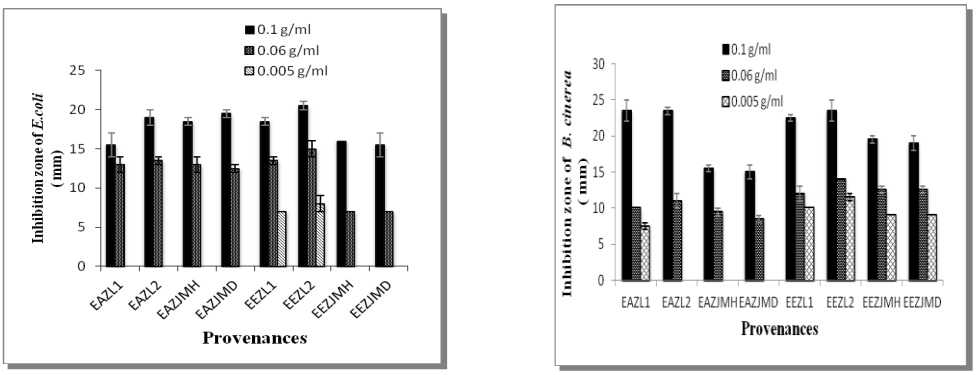


Figure 4. Linear correlation ($R^2 = 0.798$) between phenols levels and antioxydant activity of *Ziziphus lotus* and *Ziziphus jujuba* leaf extracts

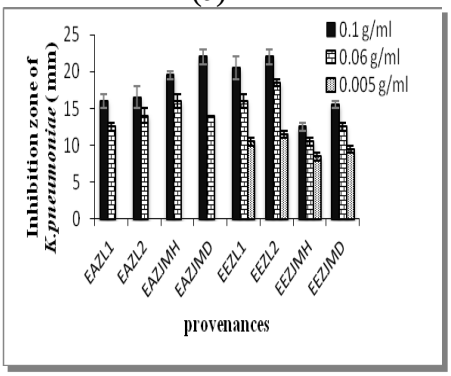
3.4. Comparative study on the effects of *Ziziphus* extracts on growth of bacterial pathogens

3.4.1. Antibacterial activity

Both aquoeus or ethanolic *Ziziphus* leaf extracts had an antibacterial activity on *E. coli* used at 0.1 et 0.06 g/ml concentrations (Figure 5a).



(a) (b)



(c)

Figure 5. Antibacterial activity of *Ziziphus* leaf extracts against *E. coli* (a), *S. aureus* (b) and *K. pneumoniae* (c) croissance's.

EEZL1: ethanolic extracts of *Z. lotus* (Kairouan), **EEZL2:** ethanolic extracts of *Z. lotus* (Rouhia) **EEZJMH:** ethanolic extracts of *Z. jujuba* (Mahres); **EEZJMD:** ethanolic extracts of *Z. jujuba* (Mahdia) **EAZL1:** aqueous extract of *Z. lotus* (Kairouan), **EAZL2:** aqueous extract *Z. lotus* (Rouhia) **EAZJMH:** aqueous extract *Z. jujuba* (Mahres), **EAZJMD:** aqueous extract *Z. jujuba* (Mahdia).

The ethanolic leaf extracts of *Z. lotus* collected from Rouhia provenance (EEZL2) had shown the highest antibacterial activity for *E. coli* with Zone of inhibition more than 20 mm (0.1 g/ml) and 15 mm (0.06 g/ml), while the 0.05 g/ml did not possess significant antibacterial activity against *E. coli* exception done for EEZL. The same tendency was observed for *S. aureus*, with inhibition zone diameter ranged between 21.5 mm (EEZL2) and 23 mm (EAZJMD) used at 0.1 g/ml concentration.

This zone of inhibition attempted the diameter of 22 mm for *K. pneumoniae* treated by EEZL2 and EAZJMD at the concentration of 0.1 g/ml. These results could confirm that the EEZL2 extract appeared to be efficient against bacterial strains. This could justify its richness on secondary metabolites especially on tannins contents. For *S. aureus*, the leaf extracts had greater inhibitor zone (23 mm) than oil fruits (11 mm). This idea was confirmed by Bukar et al. (2015). This could be explained by the richness of leaf extracts on oxygenated compounds compared to oil fruits [15].

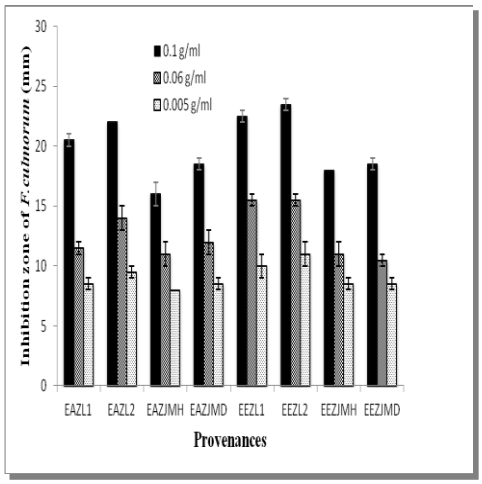
Our results were similar to these obtained by Bashir et al. (2011) who confirmed the antifungal activity of *Z. jujuba* methanolic leaf extracts. The essential oil of *Ziziphus spina-christi* fruits have been reported to have a zone of inhibition against *Escherichia coli* (10 mm). This idea was confirmed by Bukar et al. (2015). Ethanol extracts were found to be the most effective, while aqueous extracts had the moderate activity. This was idea was also identified by Medini et al. (2014).

3.4.2. Antifungal activity

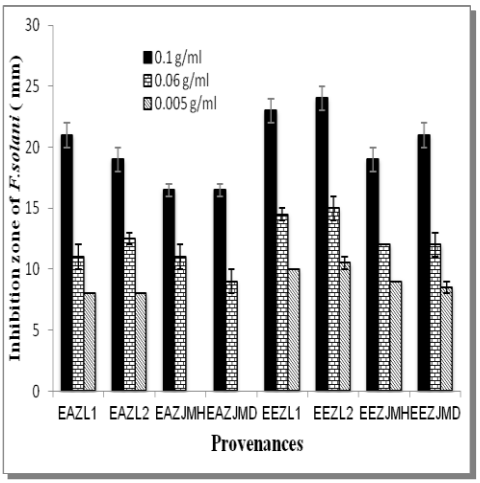
The concentration 0.1 g/ml of EEZL1 and EAZL2 showed the highest antifungal activity with the zones of inhibition of 23.5 mm, 22.5 mm and 22 mm, respectively (Figure 6).

The *F. Solani* was inhibited by all *Ziziphus* extracts used at 0.1 et 0.06 g/ml concentrations, while the EAZJMH and EAZJMD had not any antifungal activity if they were used at 0.005 g/ml concentration. The zone of inhibition varied from 16.5 to 24 mm; 9 to 15 mm and 8 to 10.5 mm for 0.1; 0.06 and 0.005 g/ml concentrations, respectively. This could be explained by the richness of theses organs on secondary metabolite especially phenols which had known by its antimicrobial activities [17]. This activity was related to the high rate of not only by the monoterpene hydrocarbons [15], but also by the tannins which bind cell walls of ruminal bacteria [1].

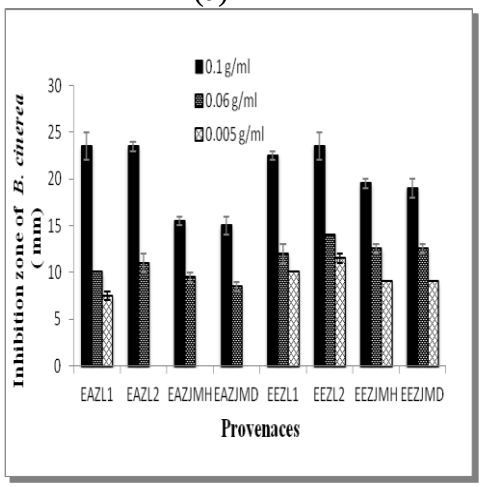
The aqueous and ethanolic leaf extracts of two studied *Ziziphus*, used at 0.1 g/ml concentration, were found the most efficient against *B. cinerea* with inhibition zones ranging from 15 mm (EAZJMD) to 23.5 mm (EEZL2, EAZL1 et EAZL2). Ethanolic leaf extracts of *Z. jujuba* showed a high antifungal activity (25 mm) on *Trichophyton rubrum* compared to aqueous extracts (19 mm) used at concentration of 10 mg/ml [18].



(a)



(b)



(c)

Figure 6. Antifungal activity of Ziziphus jujuba et Ziziphus lotus leaf extracts on F. culmorum, (a) F. Solani (b) and B. cinerea (c) crouissance's

EEZL1: ethanolic extracts of *Z. lotus* (Kairouan), **EEZL2:** ethanolic extracts of *Z. lotus* (Rouhia) **EEZJMH :** ethanolic extracts of *Z. jujuba* (Mahres) **EEZJMD:** ethanolic extracts of *Z. jujuba* (Mahdia) **EAZL1:** aqueous extract of *Z. lotus* (Kairouan), **EAZL2:** aqueous extract *Z. lotus* (Rouhia) **EAZJMH:** aqueous extract *Z. jujuba* (Mahres), **EAZJMD:** aqueous extract *Z. jujuba* (Mahdia).

4. CONCLUSION

These *in vitro* results were a first step in the search for biologically active substances of natural origin. So, others studies of the effects of aqueous and ethanol extracts of *Ziziphus* leaves would be desirable to better promote these products instead of chemicals with high degrees of toxicity to preserve the environment and improve agricultural production. An adequate toxicological study must be also carried out to verify the possibility of using these plants for fighting microorganisms.

Acknowledgements
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