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PHENOLIC COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *ZIZYPHUS LOTUS* L. AND *RUTA CHALEPENSIS* L. GROWING IN MASCARA (WESTERN ALGERIA)

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ABSTRACT

In this study, the phenolic composition, antioxidant and antimicrobial activities of *Zizyphus lotus* and *Ruta chalepensis* collected from Mascara-Western Algeria were investigated. The total phenolic, flavonoid and tannin contents in the methanolic (Me.E) and aqueous (Aq.E) extracts were measured using colorimetric methods. Polyphenolic profiles were analyzed by high performance liquid chromatography (HPLC), while the antioxidant effect was determined by DPPH* radical scavenging assays. The antimicrobial activity was estimated using agar disc diffusion and microdilution methods. The results showed that total phenolic compounds, flavonoids and tannins were significantly higher ($p < 0.05$) in *Z. lotus* extracts when compared with *R. chalepensis*. The major phenolic compound detected in *Z. lotus* was benzoic acid (1333.59 µg/g DE), while the hydroxybenzoic acid (56.60 µg/g DE), chlorogenic acid (44.60 µg/g DE), epicatechin (38.80 µg/g DE), catechin (26.30 µg/g DE) and gallic acid (13.22 µg/g DE) were the major compounds in *R. chalepensis*. Both plants were determined to be effective antioxidants regarding their lower IC_{50} values of 0.146 mg/mL for *Z. lotus* Me.E and 0.206 mg/mL for *R. chalepensis* Aq.E. Results demonstrated that Me.E of *Z. lotus* and *R. chalepensis* were more active against *S. enterica* ssp *arizonae*, while the lowest minimum inhibitory concentration was recorded against *H. alvei* using *Z. lotus* aqueous extract (25 mg/mL). An important anti-*Candida* activity was also determined. These results suggest the most efficiency of both plants, in the treatment of various human infections, regarding their potential on bioactive molecules with antioxidant and antimicrobial activities.

Keywords: *Zizyphus lotus*, *Ruta chalepensis*, Western Algeria, Phenolic compounds, HPLC-DAD, Antioxidant, Antimicrobial

INTRODUCTION

The emergence of drug resistance in various microbial species (bacteria and fungi) and the appearance of undesirable side effects for certain antibiotics are of a global health concern. In addition, the dysbiosis phenomenon resulting from the therapeutic consumption of these chemical drugs promotes the proliferation of other multidrug-resistant pathogenic germs. This renders antimicrobial therapy less effective and treatment of infectious diseases becomes more limited, thus the need for innovative approaches to tackle antimicrobial resistance **(Klein, 2018; Lange, 2016)**. Therefore, the search for alternative products isolated from medicinal plants was increasingly correlated to the problems caused by chemical synthetic drugs and most of the people, especially in developing countries, depend on plants for medicines regarding their richness on chemical bioactive constituents, such as terpenoids, phenolics, alkaloids, flavonoids, amino acids, saponins, glycosides, diterpenes and triterpenes **(Amabye *et al*., 2015)**.
Among the medicinal plants which constitute the vegetal richness of Algeria, the *Zizyphus lotus* L. and *Ruta chalepensis* L. species. *Zizyphus lotus* belonging to Rhamnaceae family is an aromatic and medicinal plant abundant in Algeria and popularly famous as "Sedra" and the edible fruit is called "Nbeg". This plant species has numerous nutritiously, cosmetically, and medicinally interests. *Z. lotus* is widely used in our region for their various properties as antioxidant, antidiabetic, dermatoprotective, antispasmolytics, anti-inflammatory and analgesic **(Marmouzi *et al*., 2019; Borgi *et al*., 2007)**. This plant is also used for the intestinal disorders and as anti-ulcerogenic **(Bakhtaoui *et al*., 2014)**. Various studies also demonstrated other biological properties of *Z. lotus* as anticancer and antibacterial (**Borgi *et al*., 2008; Benammar *et al*., 2010**). *The* fruit contains important levels of carbohydrates, vitamins, minerals, fibers, amino acids, fatty acids and phenolic compounds, which are considered the main responsible for its health benefits **(Hossain, 2018)**. **Hani *et al*. (2020)** in their study, they reported that leaf aqueous extract of *Z. lotus* has a significant antidiarrheal and anti-inflammatory activities which supports its use in traditional herbal medicine practice.
The second aromatic and medicinal plant of great interest in this study is *Ruta chalepensis* of Rutaceae family. The most diffused species in the genus *Ruta* are *Ruta chalepensis* L., *Ruta graveolens* L., and *Ruta montana* L. *Ruta chalepensis* is known for carrying various biological properties associated with its extracts and essential oils which are widely used for the treatment of gastric, diuretic, inflammatory, rheumatic disorders, and as anti-helminthic, anti-inflammatory, antioxidant, hypoglycemic, emmenagogue, spasmolytic and anti-cholinesterase, as well as an antibacterial and antifungal **(Loizzo *et al*., 2018; Günaydin and sevca, 2006; Kacem *et al*., 2015; Al-Majmaie *et al*., 2018; Bouajaj *et al*., 2014; Gali and Bedjou, 2018; Haddouchi *et al*., 2013)**. **Coimbra *et al*. (2020)** in their study on the genus *Ruta*, they confirmed that different parts of the plant are used in folk medicine to treat a wide range of different diseases, the principal use is in gynecological field, the treatment of pain, fever, nausea, inflammation, infections, nervous disorders, among others, are also described.
To the best of our knowledge, this is the first study on the antioxidant effect and the antimicrobial activity against pathogenic enteric germs of *Zizyphus lotus* and *Ruta chalepensis* growing in Mascara, western Algeria. The aim of this study was to characterize phytochemically the polyphenolic extracts (methanolic and aqueous extracts) prepared from *Z. lotus* leaves and *R. chalepensis* aerial parts (leaves, flowers and small stems) collected from Mascara- western Algeria. Various phenolic compounds have been also identified and quantified in all extracts using HPLC-DAD analysis. In addition, the antioxidant effect has been determined using DPPH* radical scavenging assays and the antimicrobial activity has been investigated using disc diffusion method on Muller-Hinton agar and microdilution technique for the determination of the minimum inhibitory, bactericidal and fungicidal concentrations (MIC, MBC and MFC).

MATERIAL AND METHODS

Plant material

The leaves of *Z. lotus* were collected during the month of July 2017, and the aerial parts (leaves, flowers and small stems) of *R. chalepensis* during the month of April 2017 from Mascara, El-Mamounia region in western Algeria and were identified by a botanist from the Department of Biology of Mascara University, Algeria. The plants were thoroughly cleaned, dried in the dark and then processed into a fine powder that was used to prepare the different phenolic extracts.

Microbial strains

Clinical isolates including Gram-positive bacteria S_1: *Staphylococcus aureus*, S_2: *Enterococcus faecalis*, the Gram-negative bacteria S_3: Enteropathogenic *Escherichia coli* (EPEC), S_4: *Salmonella enterica* ssp *arizonae*, S_5: *Proteus mirabilis*, S_6: *Hafnia alvei* and a pathogenic microscopic fungi S_7: *Candida albicans* were isolated from stool specimens of gastroenteritis patients and were identified in the Laboratory of Microbiology of the hospital Meslem Taib of Mascara, Algeria, as well as at the laboratory of Bioconversion, Microbiological Engineering and Health Safety of the department of Biology, University Mustapha Stambouli of Mascara, Algeria.

Isolation and identification of pathogenic germs

The biological samplings were carried out in the gastroenterologist service of Meslem Taib hospital of Mascara, Algeria. Fifteen patients were selected, that were suffering from gastroenteritis. Before each sample collection, patients were clinically diagnosed and the most symptoms observed were diarrhea, Nausea, fever, abdominal pains and vomiting. The stool samples were enriched in Brain Heart Infusion Broth (BHIB) and Selenite F broth (SFB), respectively and were incubated at a temperature of 37°C in order to achieve the pathogenic germ revivification. The analyses were performed for anaerobic and aerobic bacterial and fungal content by cultures on a series of selective media. Gram-positive bacteria were isolated by the streak plate method on Colombia Agar with 5% human blood media, Chapman Agar, Bile Esculin Azide Agar (BEA), while Gram-negative bacteria were isolated on Hecktoen Agar, Eosin Methylene blue Agar (EMB), MacConkey agar, Sorbitol-MacConkey agar (SMAC) and fungal strains on Sabouraud Dextrose Agar (SDA). The inoculated plates were incubated at 37°C for 24-48 h and 72 h according to the investigated strains. The microbial strains were identified to the genus level based on the colony morphology (appearance, size, margin, form, and elevation), microscopic examination (Gram's staining and motility), physiological and biochemical tests of classic technique (Triple Sugar Iron test, Kligler test, mannitol motility, Growth in hyper salty agar medium, catalase, oxidase, urease enzymes, degradation of esculin, nitrate reduction, indole production, Voges Proskauer test, citrate utilization, arginine dihydrolase (ADH) test), thus by the application of commercials kits, miniaturized multi-test systems by using API STAPH, API 20E and API CANDIDA that were applied according to the BioMerieux manual and adopting standard procedures. Pathogenicity tests were also performed: coagulase and hemolysin tests (**Kloss and Wolfshohl, 1982; Beutin *et al*., 1989**).

Antibiotics susceptibility testing

The antibiotic susceptibility test was carried out using the agar disc diffusion assay following the **CLSI guidelines, (2015)**. Susceptibility to the following antibiotics was tested: Penicillin G (10 µg/disc), Amoxycillin (25 µg/disc), Oxacillin (5 µg/disc), Neomycin (30 µg/disc), Colistin (10 µg/disc), Spiramycin (100 µg/disc), Pristinamycin (15 µg/disc), Nitroxolin (20 µg/disc) and Fluconazole (25 µg/disc) (Tab 1). Zone diameters were interpreted using the critical diameters mentioned by the **FMS-AC, (2013)** and the **FMS-AC/EUCAST, (2018)**.

Table 1 Different classes of antibiotics used.

Class	Antibiotics used
Penicillin	Penicillin G (P-G), Amoxycillin (AMX), Oxacillin (OX)
Aminoglycosides	Neomycin (N)
Polymyxin E	Colistin (CT)
Macrolide	Spiramycin (SP)
Streptogramin A	Pristinamycin (PT)
Nitroquinolone	Nitroxolin (NI)
Azole antifungal	Fluconazole (FCA)

Preparation of methanolic and aqueous extracts

Each 50 g of the fine powder of *Z. lotus* and *R. chalepensis* was separately processed by cold maceration for the preparation of the methanolic extract (Me.E) using 500 mL of methanol 80 % at 20°C under agitation for 24 h. The filtrates obtained were evaporated to dryness under vacuum using a rotary evaporator at 40°C and the methanolic extracts were stored in small glass vials at 4°C until use **(Romani *et al*., 2006)**.

The aqueous extracts (Aq.E) were prepared by decoction according to the protocol described by **Chavane *et al.* (2001)** with some modifications. For this process, the polyphenolic compounds were extracted by boiling 50 g of the fine powder of the plant in 500 mL of distilled water at 180°C under agitation for 30 min. The mixture was then filtrated and the Aq.E was stored at 4°C until use. The extraction yield expressed in percentage (%) was calculated using formula: Yield (%) = (m_2 / m_1) x 100, where m_1: weight of the plant material used (g), m_2: weight of dry extract (g).

Determination of total phenolics content (TPC)

The total phenolics content (TPC) of the extracts was determined according to **Boizot and Charpentier, (2006)**: 200 µL of each extract at a concentration of 1 mg/mL was mixed with 1 mL of Folin Ciocalteu reagent and 800 µL of sodium carbonate Na2CO3 (7.5%). The mixture was incubated in the dark and at room temperature for 10 minutes and the absorbance was determined using a spectrophotometer (JENWAY, 6400 spectrophotometer) at 735 nm. Gallic acid (GA) was used as a standard for the calibration curve ($y = 0.751x + 0.0012$, $R^2 = 0.9975$) ranged from 0.05 to 0.2 mg/mL in a methanolic solution of gallic acid. The total phenol content was expressed as gallic acid equivalents per gram of dry extract (mg GAE/g DE). All determinations were performed in triplicate.

Determination of flavonoids content (TFC)

The total flavonoids content (TFC) was determined according to **Samatha *et al.* (2012)**: 1 mL of 2% aluminum trichloride (AlCl3) methanolic solution was mixed with the same volume of the extract solution. The absorbance values were determined at 430 nm after 40 min against a blank. Quercetin (Q) was used as a standard. The concentration of the calibration curve ($y = 4.4537x + 0.0115$, $R^2 = 0.9922$) ranged from 0.05 to 0.25 mg/mL in a methanolic solution containing quercetin. The total flavonoid content of the extracts was expressed in mg of quercetin per gram of dry extracts from *Z. lotus* leaves and *R. chalepensis* aerial parts (mg QE/g DE).

Determination of total tannins content (TTC)

The total tannins content (TTC) was determined by the vanillic acid method described by **Ba *et al.* (2011)**: The vanillin reagent was prepared by mixing equal volumes: 8% (v/v) HCL, 37% (v/v) methanol and 4% vanillin in methanol (w/v). The mixture was maintained at 30°C before the assay. Volumes of 200 µL for each extract were added to 1000 µL of vanillin reagent. The mixture was incubated for 20 min and the absorbance was determined at 500 nm. Catechin was used as a standard and the calibration curve ($y = 0.1117x + 0.0014$, $R^2 = 0.9989$) comprised a concentration range from 0.5 to 2 mg/mL in a methanolic solution of catechin. The total tannin content was expressed as mg of catechin equivalent per gram of dry extracts (mg CE/g DE).

High performance liquid chromatography (HPLC-DAD)

The phenolic composition analyses of different extracts were made according to **Caponio *et al.* (1999)** with slight modifications and were performed using an HP-Agilent 1290 Infinity HPLC equipped with a C18 column and diode array detector (DAD) as a detector. As a mobile phase, 3% acetic acid in (A) water and methanol (B) was used. Injection volumes were 10 µL and extract concentrations were 20 mg/mL. The samples were detected at 278 nm. The samples were prepared in methanol, and injection volumes were 20 µL. The elution gradient applied at a flow rate of 0.8 mL/min was: 93% A-7% B (0.1 min), 72% A-28% B (20 min), 75% A-25% B (8 min), 70% A-30% B (7 min) and the same gradient for 15 min was 67% A-33% B (10 min), 58% A-42% B (2 min), 50% A-50% B (8 min), 30% A- 70% B (3 min), 20% A-80% B (2 min) and 100% B in 5 min until the end of the run. Gallic acid, (+)-catechin, chlorogenic acid, *p*-hydroxybenzoic acid, (-)-epicatechin, *p*-coumaric acid, syringic acid, ferulic acid, sinapinic acid, benzoic acid, hesperidin, rosmarinic acid, *trans*-cinnamic acid and quercetin were used as standards. Identification and quantitative analysis were done by comparison with standards. The amount of each phenolic compound was expressed as µg per gram of the extract using external calibration curves, which were obtained for each phenolic standard.

Antioxidant effect using DPPH* radical scavenging activity

The scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH*) free radical was used for the evaluation of the antioxidant activity and determined using method described by **Blois, (1958)**. One mL of the sample solutions were added to 4 mL of DPPH* ethanol solution (0.2 mg/mL), and the mixtures were incubated in dark at 20°C. After 30 min, the absorbance was measured at 517 nm with a spectrophotometer (UV 2600-Shimadzu). The antiradical activity was

calculated using the formula: Inhibition (%) = [(A control−A sample) / A control] x 100, where A control is the absorbance of the DPPH* solution and A sample is the absorbance of the DPPH* solution after the addition of the sample concentrations. Butylated hydroxyanisole (BHA) and Trolox were used as standard antioxidants. Assays were carried out in triplicate and the results were expressed as mean values ±standard deviations (SD). The values of scavenging effect were expressed as IC_{50} (mg/mL) which corresponding to the extract concentration resulting in a 50% of DPPH* inhibition determined from the inhibition curves. The results were compared to the standard antioxidants used. A higher DPPH* radical scavenging activity was associated with a lower IC_{50} values.

Antimicrobial activity tests

Disc diffusion assays

The disc diffusion method was employed to determine qualitatively the antimicrobial effect of the polyphenolic extracts (methanolic and aqueous extracts) of *Z. lotus* and *R. chalepensis*. Firstly, Muller-Hinton agar (MHA) plates were spread by culture suspensions adjusted to 0.5 McFarland. Microbial strains were adjusted to a final density of 10^8 germs/mL at 620 nm for bacteria (JENWAY, 6400 spectrophotometer) and 10^7 cells/mL at 450 nm for yeast **(Noumi *et al*., 2010)**. Therefore, sterile discs were impregnated separately by 20 µL of each methanolic and aqueous extract solutions (at a concentration of 200 mg/mL) and were placed on the inoculated plates. These extracts were dissolved in dimethyl sulfoxide (DMSO) 10% that was used as a negative control. After 2 h at 4°C in order to ensure the pre-diffusion of the extracts, plates were incubated at 37°C for 24 h and inhibitory effect was expressed by measuring diameters of growth inhibition zones (ø). All assays were carried out in triplicate. The assessment of polyphenolic extracts effectiveness was made according to **Ponce *et al*. (2003)**: Ø < 8mm: Resistant, 9 mm < Ø < 14 mm: Sensitive, 15 mm < Ø < 19 mm: Very sensitive, Ø >20 mm: Extremely sensitive.

Microdilution assays

The assessment of the antimicrobial activity by microdilution method was performed to determine the minimum inhibitory, bactericidal and fungicidal concentrations (MIC, MBC and MFC) in sterile 96-well microplates and it was carried out according to the method provided by **Chandrasekaran *et al*. (2004)**. Briefly, 50 µL of Mueller Hinton broth (for bacteria test) or Sabouraud broth (for yeast test) was placed into wells of the microplates. Subsequently, 50 µL of each Me.E and Aq.E at concentration of 200 mg/mL was added to the first column and then serial dilutions were obtained even to achieve final concentration of 1.56 mg/mL, and then 50 µL of the adjusted microbial suspensions (0.5 McFarland) were inoculated in each microplate well. The Microplates were incubated at 37°C and microbial growth kinetics were measured by reading the optical density at 620 nm for bacteria and 450 nm for fungi at 0-2-18-24-48 and 72 h, using a Microplate Absorbance Reader (Tecan Spectra II Microplate Reader). The microbial tests were prepared in triplicate. For the determination of the MBC and MFC values, each dilution starting with that representing the MIC value, was spread on MHA, incubated at 37°C for 24 h and the viable bacteria and fungi cells were counted. The dilution for which no bacterial or fungal colony was counted represents the MBC and the MFC. Generally, the values of MBC and MFC are superior or equal to the MIC values.

Statistical analysis

Replicates were prepared for all experiments. The results were given as means and their standard deviations (means ±SD). The means were compared by using the one-way and multivariate analysis of variance (ANOVA). The differences between individual means were deemed to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of the bacterial strains

The API system, physiological and biochemical tests allowed us to identify 7 strains isolated from stool specimens of gastroenteritis patients. The biochemical characteristics of the microbial isolates varied depending on the strain. While the API 20E test was used for Gram-negative bacteria, the API STAPH, coagulase and hemolytic activity tests was used for *Staphylococcus aureus* identification and the API CANDIDA for fungi strains: *Candida albicans*. The hemolytic activity test was also used for the confirmation of enteropathogenic *Escherichia coli* identification.

Antibiotics susceptibility testing

Results of the antibiotic susceptibility testing are mentioned in Table 5. The *in vitro* sensitivity of strains was done against multiple antibiotics. According to the **FSM-AC, (2013)** and the **FMS-AC/EUCAST, (2018)**, the results indicated that all the clinical strains (Gram-positive, Gram-negative bacteria and *C. albicans* strains) isolated for the antimicrobial assays assessment were resistant to most antibiotics used, which led us to qualify these clinical isolates as being pathogenic, multi-drug resistant strains.

Table 2 Antibiotic susceptibility profiles of the pathogenic bacterial and fungal strains

	Diameters of growth inhibition zones for the different clinical microbial strains using the antibiotics (ø mm)								
Microbial strains	SP (100 µg)	AMX (25 µg)	PT (15 µg)	NI (20 µg)	N (30 µg)	OX (5 µg)	CT (10 µg)	P (10 µg)	FCA (25 µg)
S_1	0^R	12^R	0^R	18^I	15^I	0^R	12^R	10^R	0^R
S_2	23^S	19^I	20^I	12^I	0^R	0^R	0^R	21^I	0^R
S_3	0^R	0^R	0^R	20^I	15^R	0^R	11^R	0^R	0^R
S_4	0^R	0^R	0^R	22^I	18^S	0^R	13^R	0^R	0^R
S_5	0^R	15^R	0^R	14^I	16^R	0^R	0^R	10^R	0^R
S_6	0^R	0^R	0^R	20^I	20^S	0^R	13^R	0^R	0^R
S_7	0^R	0^R	0^R	0^R	0^R	0^R	0^R	0^R	0^R

Legend: SP: Spiramycin, AMX: Amoxycillin, PT: Pristinamycin, NI: Nitroxolin, N: Neomycin, OX: Oxacillin, CT: Colistin, P: Penicillin-G, FCA: Fluconazole, S_1: *S. aureus*, S_2: *E. faecalis,* S_3: *E. coli,* S_4: *S. enterica* ssp *arizonae,* S_5: *P. mirabilis,* S_6: *H. alvei,* S_7: *C. albicans*, R: Resistant, S: Sensitive, I: Intermediate sensitivity.

Extraction yield, total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents

In this study, we aimed to highlight the differences in biologically active compounds between the aerial parts of *R. chalepensis* and the leaf extracts of *Z. lotus*. The extraction yields, the total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents are summarized in table 3. According to the obtained results, the aqueous extracts recorded significantly higher yields ($p < 0.05$) compared to the methanolic extracts for both plants. The highest yield was found with the Aq.E of *Z. lotus* (30.4 ±0.26%) followed by *R. chalepensis* (24.16 ±0.8%) (Tab 3). These values were significantly ($p < 0.05$) greater than those found for the Me.E, with yields of 15.57 ±0.025% and 13.2 ±0.2% for *Z. lotus* L. and *R. chalepensis* L., respectively. In addition, *Z. lotus* gave higher Me.E and Aq.E yields than *R. chalepensis*.

The total phenolics, flavonoids and tannins content varied significantly ($p < 0.05$) among the studied plants in the methanolic and aqueous extracts. Significantly higher amounts ($p < 0.05$) of phenolic compounds were determined and quantified in *Z. lotus* Extracts. The total phenolic, flavonoid and tannin contents were about 233.5 ±0.16 mg GAE/g DE, 149.87 ±0.12 mg QE/g DE and 108.5 ±0.044 mg CE/g DE, respectively in the methanolic extract, and 233.5 ±0.43 mg GAE/g DE, 124.72 ±0.15 mg QE/g DE and 112.08 ±0.021 mg CE/g DE, respectively in the aqueous extract (Tab 3). Similar concentrations on total phenols (233.5 ±0.43 mg GAE/g DE) were determined in both Me.E and Aq.E of *Z. lotus*. However, *R. chalepensis* extracts presented slight lower levels of these contents when compared with *Z. lotus*. The Me.E and Aq.E of *R. chalepensis* showed phenolic composition with higher values: 198.88 ±0.076 mgGAE/g DE, 163.46 ±0.081 mg GAE/g DE, respectively on phenol content (Tab 3).

Table 3 Extraction yields, total phenolic (TPC), flavonoid (TFC) and tannin (TTC) contents expressed in mg per gram of dry extract (mg/g DE) calculated from the different extracts of *Z. lotus and R. chalepensis*. Measurements were performed in triplicate. Results are expressed as means ±SD. p <0.05.

Extracts	Yield (%)	TPC (mg GAE/g DE)	TFC (mg QE/g DE)	TTC (mg CE/g DE)
ZLMe.E	15.57 ±0.025	233.5 ±0.16	149.87 ±0.12	108.5 ±0.044
ZLAq.E	30.4 ±0.26	233.5 ±0.43	124.72 ±0.15	112.08 ±0.021
RCMe.E	13.2 ±0.2	198.88 ±0.076	67.69 ±0.015	18.97 ±0.0025
RCAq.E	24.16 ±0.81	163.46 ±0.081	43.89 ±0.022	17.18 ±0.0051

In addition, a total flavonoids content of 67.69 ±0.015 mg QE/g DE and 43.89 ±0.022 mg QE/g DE were quantified in the methanolic and aqueous extracts of *R. chalepensis*, respectively. Also, we recorded a lower concentrations on tannins content in *R. chalepensis* methanolic and aqueous extracts, with values of 18.97 ±0.0025 mg CE/g DE and 17.18 ±0.0051 mg CE/g DE, respectively. For both plant extracts, the significant highest amounts (p <0.05) on phenols, flavonoids and tannins were obtained for the methanolic extracts, which explained that the use of methanol, as an organic solvent is more efficient for the extraction of high amounts on phenolic compounds.

The results indicated that both plants showed significant higher concentrations (p <0.05) of these bioactive molecules comparing with other studies, in which lower phenolic contents have been determined **(Neffati *et al*., 2017)**. All these results were in agreement with those of **Chetibi and Diab, (2016)**, in which the phytochemical analysis made on the extracts prepared from different parts of *Z. lotus* have given positive results. Furthermore, our results were more interesting than those obtained by **Belmaghraoui *et al*. (2018)**, who have determined a total phenol and flavonoid contents of 143.12 mg GAE/mL and 4.281 mg Rutin Equivalent (RE)/mL, respectively, in methanolic extract of *Z. lotus* fruits collected from Zaouiat Cheikh area, near Oued Zem City, Morocco. The Me.E and Aq.E of *R. chalepensis* showed total phenols composition with higher values than those obtained by **Khadri *et al*. (2016)**. Thus, according to **Loizzo *et al*. (2017)**, the Me.E of *R. chalepensis* from Italy has shown a total phenol, flavonoid and tannins with values of 6.22 mg GAE/g DE, 6.59 mg QE/g DE and 0.72 mg EG/g DE, respectively. According to **Gonzalez Trujano *et al*. (2006)**, *R. chalepensis* was known for its richness in flavonoids, phenols and tannins, which was consistent with our results (Tab 3). Moreover, **Fakhfakh *et al*. (2012)** have revealed total phenols content in aqueous extract of 51.28 mg GAE/g E for *R. chalepensis* collected in Tunisia. **Ereifej *et al*. (2015)** have also reported a total phenols and tannins content of 1328.8 and 27.8 mg/100 g E, respectively, for *R. chalepensis* leaves collected in Jordan. Comparing with our results, we concluded that plants collected from Mascara in western Algeria were much richer in active substances compared to plants from other regions and countries, and each of phenolic compounds and flavonoids contribute widely to human health by their biological properties **(Ghasemzadeh, 2011)**.

Identification and quantification of various phenolic compounds (HPLC-DAD)

Ruta chalepensis and *Zizyphus lotus* are a medicinal plants representing an interesting chemical composition and used in traditional medicine to treat a wide range of pathologies. Results of chromatogram profiles and concentrations of phenolic compounds identified are shown in Fig 1-2-3-4 & Table 4 respectively. Various phenolic compounds were identified using HPLC-DAD analysis in *Z. lotus* and *R. chalepensis* extracts and included phenolic acids with hydroxycinnamic acids and hydroxybenzoic acids. In addition, flavonoids were also quantified and identified: catechin, epicatechin, quercetin and hesperidin. Higher benzoic acid level around 1333.59 µg/g DE was quantified in the methanolic extract of *Z. lotus* leaves, compared with the aqueous extract (42.58 µg/g DE) (Tab 4).

Table 4 Phenolic compounds (µg/g DE) identified in methanolic and aqueous extracts of *Z. lotus* leaves and *R. chalepensis* aerial parts collected from Mascara, western Algeria.

N°	Phenolic compounds	R_t (min)	ZLMe.E	ZLAq.E	RCMe.E	RCAq.E
1	Gallic acid	5.400	1.27	4.39	13.22	12.25
2	Catechin	12.430	8.24	4.60	26.30	22.54
3	Chlorogenic acid	15.745	2.91	1.53	44.66	1.55
4	Caffeic acid	18.336	ND	ND	2.50	1.65
5	Hydroxybenzoic acid	18.917	ND	ND	56.60	ND
6	Epicatechin	19.165	ND	ND	38.80	24.01
7	Syringic acid	21.250	0.73	ND	1.11	0.56
8	Coumaric acid	26.385	ND	ND	2.26	ND
9	Trans-Ferrulic acid	31.265	ND	ND	ND	ND
10	Sinapic acid	33.416	ND	ND	ND	ND
11	Benzoic acid	38.571	1333.59	42.58	ND	ND
12	Hesperidin	54.719	ND	ND	ND	ND
13	Rosmarinic acid	59.326	ND	ND	ND	ND
14	Cinnamic acid	68.506	1.00	ND	ND	ND
15	Quercetin	71.045	ND	ND	1.33	4.22

Legend: N°: Number, R_t (min): Retention time (minute), ZLMe.E: *Z. lotus* methanolic extract, ZLAq.E: *Z. lotus* aqueous extract, RCMe.E: *R. chalepensis* methanolic extract, RCAq.E: *R. chalepensis* aqueous extract, ND: Not detected.

Catechin, chlorogenic acid, gallic acid, cinnamic acid and syringic acid were also detected with low concentrations in the methanolic and aqueous extracts of *Z. lotus*. Moreover, nine phenolic compounds were identified in *R. chalepensis* methanolic extract. These compounds included six phenolic acids (gallic acid, chlorogenic acid, caffeic acid, hydroxybenzoic acid, syringic acid and coumaric acid) and 3 flavonoids: catechin, epicatechin and quercetin. The hydroxybenzoic acid (56.60 µg/g DE), chlorogenic acid (44.66 µg/g DE), epicatechin (38.80 µg/g DE), catechin (26.30 µg/g DE) and gallic acid (13.22 µg/g DE) were the major components detected. The gallic acid, catechin and epicatechin represented the major compounds of phenols in the aqueous extract of *R. chalepensis*, whereas the chlorogenic acid was weakly represented with a concentration of 1.55 µg/g DE, compared with the methanolic extract (Tab 4). Furthermore, the HPLC-DAD results revealed that *R. chalepensis* extracts exhibited higher flavonoid levels than *Z. lotus*.

Moreover, both *R. chalepensis* extracts were rich in catechin and epicatechin. Variations were detected between the methanolic and aqueous extracts for each plant, hydroxybenzoic acid and coumaric acid were detected in Me.E of *R. chalepensis*, while it were absent in the aqueous extract. Syringic acid and cinnamic acid were detected in *Z. lotus* methanolic extract, but absent in the Aq.E. According to **Wei *et al*. (2018),** benzoic acid, which was detected in *Z. lotus* with higher concentrations, is largely described for its antimicrobial activities. The content variations on bioactive components in each plant extract is correlated to the extraction solvent used, its concentration and the time used for the polyphenol extraction process **(Benchikh *et al*., 2018)**. In accordance with our results, recent studies carried out by **Marmouzi *et al*. (2019)** and **Ouerghemmi *et al*. (2016)** have shown that the extracts of *Z. lotus* and *R. chalepensis*, respectively, contains various phenolic compounds with a high quantity of phenolic acids and flavonoids.

In addition, our results suggested that phenolic acids and flavonoids conferred an important value to *Ruta* chalepensis leaves, flowers and small stems, which is in agreement with results of **Kacem *et al*. (2015)**. **Mkadmini Hammi *et al*. (2017)** have revealed that fruit extract from *Z. lotus* collected from Tozeur, South of Tunisia are rich in flavonoids, with quercetin the most dominant compound in the sample (27.69 mg/L), catechin the second most abundant (20.7 mg/L) followed

by gallic acid (7.55 mg/L), kaempferol (3.28 mg/L) and syringic acid (1.37 mg/L). In their study, **Tlili *et al*. (2019)** have determined the highest amount of phenolic compounds in *Z. lotus* from Tunisia (1087.8 mg/g). On the other hand, our results suggested that phenolic compounds identified and quantified during this study can award an important value to *Z. lotus* leaves and the aerial parts (leaves, flowers and small stems) of *R. chalepensis*, collected from El-Mamounia region of Mascara-western Algeria, which may therefore be an alternative source of natural substances, used as antioxidants and antimicrobials.

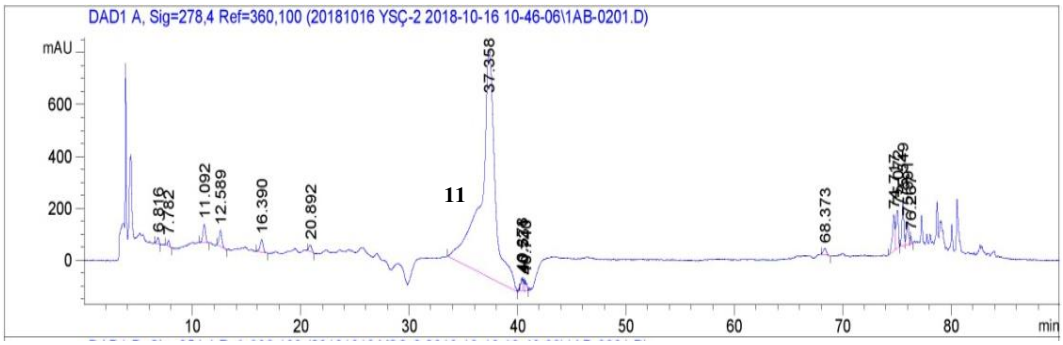

Figure 1 HPLC-DAD phenolic profile of *Z. lotus* methanolic extract detected at 278 nm. 11: Benzoic acid.

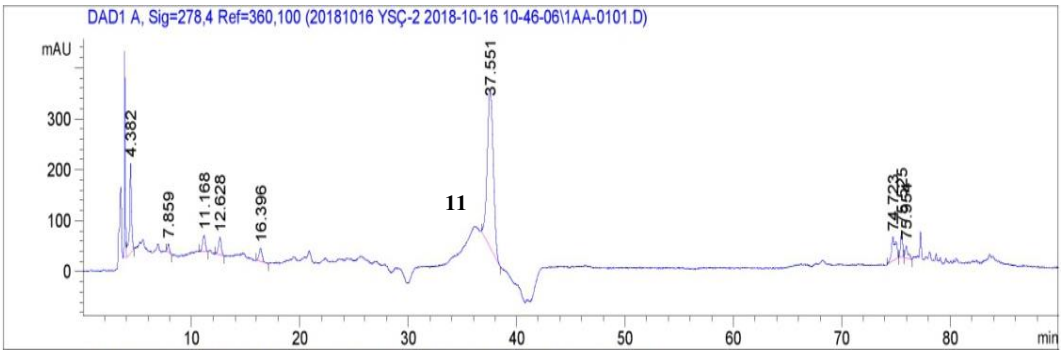

Figure 2 HPLC-DAD phenolic profile of *Z. lotus* aqueous extract detected at 278 nm. 11: Benzoic acid.

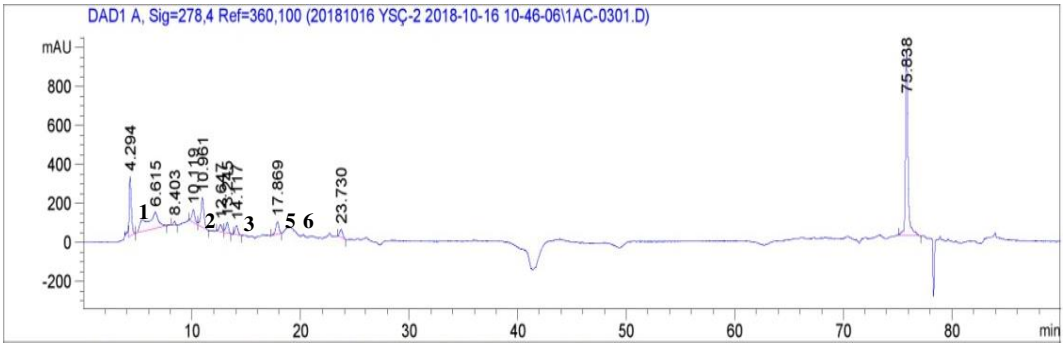

Figure 3 HPLC-DAD phenolic profile of methanolic extract of *R. chalepensis* aerial parts detected at 278 nm. 1: Gallic acid, 2: Catechin, 3: Chlorogenic acid, 5: Hydroxybenzoic acid, 6: Epicatechin.

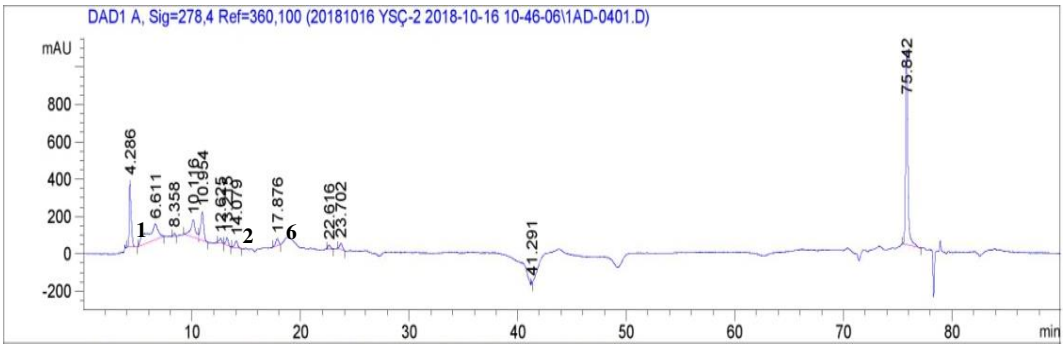

Figure 4 HPLC-DAD phenolic profile of aqueous extract of *R. chalepensis* detected at 278 nm. 1: Gallic acid, 2: Catechin, 6: Epicatechin.

Antioxidant effect using DPPH* Free radical scavenging assay

The DPPH* scavenging activity results of the methanolic and aqueous extracts of *Z. lotus* and *R. chalepensis*, and the standard antioxidants used (BHA and Trolox) expressed as the percentage of the DPPH* free radicals inhibition and the IC_{50} values (mg/mL) are mentioned in Table 5. According to the obtained results, the methanolic and aqueous extracts of *Z. lotus* leaves and *R. chalepensis* aerial parts were significantly more effective ($p < 0.05$) as free radical scavengers comparing to standards compounds used (BHA and Trolox).

Table 5 Scavenging effect on DPPH* free radical (percentage %) and Inhibition DPPH* concentration (IC_{50}) values (mg/mL) of *Z. lotus* and *R. chalepensis* extracts. Results are expressed as means ±SD that were calculated from triplicate assays. P <0.05: Significant effect.

Polyphenolic extracts	Polyphenolic extract concentrations (mg/mL)					
	0.005	0.015	0.025	0.035	0.05	IC_{50}
ZLMe.E	56.51 ±1.61	62.32 ±0.45	83.44 ±0.73	88.53 ±0.38	88.81 ±0.18	0.146
ZLAq.E	53.30 ±0.31	62.93 ±0.28	82.69 ±0.29	87.56 ±0.47	87.72 ±0.72	0.342
RCMe.E	51.98 ±1.2	59.16 ±2.95	85.85 ±0.67	88.36 ±0.68	87.59 ±0.40	0.551
RCAq.E	53.77 ±0.43	65.43 ±3.37	85.25 ±5.62	90.42 ±0.26	89.29 ±0.6	0.206
Trolox	51.32 ±0.24	67.58 ±0.45	88.93 ±0.72	90.05 ±0.3	90.49 ±0.80	0.255
BHA	51.88 ±0.31	69.35 ±7.80	90.35 ±0.24	90.20 ±0.46	90.91 ±0.64	0.115

All extracts showed an important free radical scavenging activity at low concentration (0.005 mg/mL) than standard compounds used (BHA= 51.88 ±0.31% and Trolox= 51.32 ±0.24%) (Tab 5). A statistical significant difference (p <0.05) was found among the extract samples. *Z. lotus* Me.E was more efficient at this concentration with a significant scavenging effect (p <0.05) on DPPH* free radicals percentage of 56.51 ±1.61%. The extracts of *Z. lotus* leaves and the aerial parts (leaves, flowers and small stems) of *R. chalepensis* exhibited the greatest activity, while *R. chalepensis* Me.E had the lowest activity at low concentrations (0.005 mg/mL = 51.98 ±1.2%, 0.015 mg/mL = 59.16 ±2.95%) (Tab 5). At a concentration of 0.035 mg/mL, all the extracts expressed inhibition percentages of DPPH* that were close to the BHA and Trolox values (90.20 ±0.46 and 90.05 ±0.3%, respectively). Excellent scavenging effects with percentages of 88.81±0.18% and 89.29 ±0.6% were observed with *Z. lotus* Me.E and *R. chalepensis* Aq.E at 0.05 mg/mL, respectively. These extracts were more efficient in the reduction of DPPH* with a lower IC_{50} values (0.146 and 0.206 mg/mL, respectively). Moreover, all the studied phenolic extracts were deemed with an excellent significant antioxidant effect (p <0.05).

Phenolic compounds were the most studied for their antioxidant activities that protect from many diseases by their capacity to neutralize free radicals **(Jayaprakasha *et al*., 2001)**. More recently, **Saiah *et al*. (2016)** have reported an important antioxidant activity of *Z. lotus* extracts which increased with the concentration increase of the extracts. **Khouchlaa *et al*. (2020)**, they showed that the methanolic extract of *Z. lotus* was more active against free radicals of DPPH*, with IC_{50} of 5 mg/mL. During the present study, IC_{50} values were recorded much lower than those reported in the literature. Therefore, our results were in agreement with studies of **Gali and Bedjou, (2018)** in which they have determined that *R. chalepensis* exerts a good antioxidant effect with lower IC_{50} values. According to the results obtained during this study, we observed that both plant extracts exhibited very promising antioxidant activities close to BHA and Trolox, regarding their potential on DPPH* free radical scavenging, with a low IC_{50} values for all samples, which can be attributed to the presence of potent phenolic compounds in *Z. lotus* leaves and *R. chalepensis* aerial parts collected from Mascara, western Algeria.

Antimicrobial activity assays

Results of the antimicrobial effect assessment mentioning the microbial sensitivity profiles of the clinical isolates (Gram-positive, Gram-negative bacteria and fungi strains) to the prepared natural drugs (Me.E and Aq.E of both plants) according to **Ponce *et al*. (2003)** and the minimum inhibitory, bactericidal and fungicidal concentrations are shown in Table 6 & 7. The results of the *in vitro* antimicrobial assay revealed that the extracts of *Z. lotus* leaves and *R. chalepensis* aerial parts possessed great potential for antibacterial and anti-*Candida* activities (Tab 6). The dimethyl sulfoxide (DMSO 10%) used as negative control did not exhibit neither antibacterial nor antifungal effect against the assayed microbial strains. With reference to the antimicrobial activity to various antibiotics used, the phenolic extracts of both plants were more active, but the inhibitory potency of *R. chalepensis* was less than *Z. lotus* extracts. *C. albicans* showed sensitivity to all extracts of both plants with growth inhibition diameters exceeding 10 mm. The methanolic and aqueous extracts of *Z. lotus* and *R. chalepensis* were more active against all Gram-positive, Gram-negative bacteria and *C. albicans* with its significant effects (p <0.05), while enteropathogenic *E. coli* and *S. enterica* ssp *arizonae* were the most sensitive with inhibition diameters of 16.1 ±0.1 and 38.06 ±0.1 mm, respectively, for *Z. lotus* Me.E.

The aqueous extract of *Z. lotus* exhibited a significant bactericidal activity (p <0.05) with diameters of 17.03 ±0.08 and 25 mm for *E. coli* and *S. enterica* ssp *arizonae,* respectively. *S. aureus* and *E. faecalis* were sensitive to all the extracts of both plants, with inhibition diameters ranged for 9 to 19mm. For Gram-negative bacteria, some strains were distinguished by a very high sensitivity compared to others, as shown by the case of *S. enterica* ssp *arizonae* and *H. alvei* whose inhibition diameters were much higher by applying *Z. lotus* extracts comparing to the potency effect of *R. chalepensis*.

Table 6 Antimicrobial activity of the methanolic and aqueous extracts from Algerian *Zizyphus lotus* and *Ruta chalepensis* against enteropathogenic clinical germs. The values are presented as the mean of three replicates ±the standard deviation. P <0.05: Significant difference.

Clinical strains	Diameters of growth inhibition zones for the different clinical microbial strains using plant extracts (ø mm)				
	ZLMe.E	ZLAq.E	RCMe.E	RCAq.E	DMSO
S_1	11.1 ±0.1 ^{S}	14.03 ±0.06 ^{S}	10.1 ±0.1 ^{S}	9.07 ±0.1 ^{S}	NE
S_2	9.03 ±0.06 ^{S}	7.1 ±0.1 ^{R}	19.03 ±0.2 ^{HS}	NE ^{R}	NE
S_3	16.1 ±0.1 ^{HS}	17.03 ±0.08 ^{HS}	9.03 ±0.06 ^{S}	NE ^{R}	NE
S_4	38.06 ±0.1 ^{EHS}	25 ±0 ^{EHS}	20.13 ±0.2 ^{EHS}	17.1 ±0.1 ^{HS}	NE
S_5	20 ±0 ^{EHS}	25 ±0 ^{EHS}	11.03 ±0.06 ^{S}	NE ^{R}	NE
S_6	23.03 ±0.06 ^{EHS}	32.1 ±0.1 ^{EHS}	12.03 ±0.06 ^{S}	6.03 ±0.06 ^{R}	NE
S_7	13.2 ±0.2 ^{S}	10.06 ±0.1 ^{S}	12.33 ±0.6 ^{S}	10.33 ±0.6 ^{S}	NE

Legend: ø (mm): Diameters of growth inhibition zone in millimeter, S_1: *S. aureus*, S_2: *E. faecalis,* S_3: *E. coli,* S_4: *S. enterica* ssp *arizonae,* S_5: *P. mirabilis,* S_6: *H. alvei,* S_7: *C. albicans*, NE: No effect. R: Resistance (Ø < 8mm), S: Susceptibility (9 mm< Ø <14 mm, HS: High susceptibility (15 mm< Ø <19 mm), EHS: Extremely high susceptibility (Ø >20 mm).

Table 7 Antimicrobial parameters as minimum inhibitory, bactericidal and fungicidal concentrations (MIC, MBC and MFC) against each microbial strain tested.

Tested strains		Minimum inhibitory, bactericidal and fungicidal concentrations (mg/mL) expressed as MIC-MBC and MIC-MFC			
		ZLMe.E	ZLAq.E	RCMe.E	RCAq.E
S_1	MIC-MBC	100-200	100-100	50-100	200-200
S_2	MIC-MBC	100-200	50-100	50-100	100-200
S_3	MIC-MBC	100-200	100-200	100-200	200-200
S_4	MIC-MBC	100-100	50-200	50-100	100-100
S_5	MIC-MBC	100-100	200-200	100-200	200-200
S_6	MIC-MBC	100-100	25-100	100-200	100-200
S_7	MIC-MFC	100-100	50-100	50-100	50-100

Legend: S_1: *S. aureus*, S_2: *E. faecalis,* S_3: *E. coli,* S_4: *S. enterica* ssp *arizonae,* S_5: *P. mirabilis,* S_6: *H. alvei,* S_7: *C. albicans*.

The greatest significant antimicrobial effect ($p < 0.05$) of *R. chalepensis* on *S. enterica* was recorded using Me.E with diameters of growth inhibition zone of 20.13 ±0.2 mm. All the clinical strains were sensitive to the polyphenolic extracts of *R. chalepensis*, while no antibacterial effects against *E. coli* and *P. mirabilis* were determined using the aqueous extract (Tab 6). For both plants, results were confirmed by the MIC, MBC and MFC values (Tab 7). The inhibitory effect was observed to be correlated to the concentrations of the phenolic extracts and according to the tested bacterial and fungal strains. The inhibitory properties of the methanolic and aqueous extracts were observed within a range of concentrations from 25-to100 mg/mL.

The lowest MIC values were observed against *H. alvei* when applying the aqueous extract of *Z. lotus* at a concentration of 25 mg/mL. The inhibitory effect of *Z. lotus* against all microorganisms tested could be explained by the most abundant richness of *Z. lotus* leaves in phenols, especially the benzoic acid known as a potent antimicrobial, which accelerated the rate of bacterial and fungal mortality. Gallic acid, chlorogenic acid, caffeic acid, hydroxybenzoic acid, epicatechin, syringic acid, coumaric acid and quercetin detected in *R. chalepensis* extracts were the phenolic compounds that exhibited antibacterial and antifungal effects. Results varied according to the technique used for the antimicrobial potency assessment. The results obtained in liquid medium were higher for some microbial strains than those in agar medium, as shown by the case of *E. faecalis* that showed sensitivity to the Aq.E of *R. chalepensis* in liquid medium, with an MIC value of 100 mg/mL (Tab 7).

Very effective antimicrobial activity with bactericidal and fungicidal properties was observed at a concentrations ranging from 100 to 200 mg/mL against all the clinical bacteria and yeast tested during this study. The MFC values of *Z. lotus* and *R. chalepensis* extracts were ranged from 25 to 100 mg/mL, according to the tested extract. Results suggested that phenols, tannins and flavonoids contained in both plants extracts exhibited an increase of toxicity to the pathogenic microorganisms.

Our results were in agreement with those of **Alotaibi *et al.* (2018)** and **Daoudi *et al.* (2016)** who have determined an antifungal effect of various extracts of *R. chalepensis*. Furthermore, our results were superior to those obtained by **Kasimala *et al.* (2014)** who have showed that *R. chalepensis* Aq.E exhibit a weak antibacterial effect on *S. aureus*. The studies of **Boumediene, (2012)** have indicated that *R. chalepensis* has no effect against *E. coli*, which is in agreement with the results obtained during this study. Regarding the antimicrobial potency of polyphenolic extracts of *Z. lotus* leaves and comparing our results with other studies, the Aq.E prepared from the leaves of this plant collected from Mascara, El-Mamounia region, western Algeria has exerted a very effective and superior antimicrobial effect in comparison with the results of **Hamza and Meziani, (2015)**. In addition, our results were in accord with those of **Elaloui *et al.* (2017)**, **Lahmer and Messai, (2017)** who have shown that *Z. lotus* leaf extracts have a greater antibacterial effect on *S. aureus* and *E. coli* and more recently, **Saiah *et al.* (2016)** have reported an important antibacterial activity of *Z. lotus* extracts. Many studies indicated that phenolic compounds and condensed tannins (catechin and epicatechin) are endowed of antibacterial and fungicidal effects and could bind to bacterial cell walls **(Cowan, 1999; Bukar *et al.*, 2015)**.

CONCLUSION

The total phenol, flavonoid and tannin contents, the antioxidant effect and the antimicrobial activity of *Z. lotus* leaf and *R. chalepensis* aerial parts (leaves, flowers and small stems) growing in Mascara, western Algeria have been investigated in this study for the first time, against pathogenic clinical bacteria and yeast isolated from patients with gastroenteritis. We detected the presence of various bioactive compounds in all extracts using HPLC-DAD analysis. In addition, quantitative analysis showed a very high amounts on total phenol, flavonoid and tannin contents in *Z. lotus* and *R. chalepensis* extracts. *Z. lotus* leaves was the most rich in these bioactive substances compared with *R. chalepensis*, which explain the highest potency of the bactericidal and fungicidal effects against all the clinical microorganisms tested. *Zizyphus lotus* and *Ruta chalepensis* collected from Mascara, western Algeria were found to be more efficient against Gram-positive, Gram-negative bacteria and *Candida albicans* than standard antibiotics used in this study. A highest antioxidant activity of both plant extracts was observed with lower IC_{50} values compared with standard antioxidants. *Zizyphus lotus* and *Ruta chalepensis* were found to be more efficient as BHA and Trolox compounds. Our results indicated that the biological activities depend on the component type of these extracts, as well on the plant part and the harvest area, suggested for a better valorization of *Z. lotus* and *R. chalepensis* from western Algeria, and for a further investigation on others potent antioxidant and antimicrobial molecules from these medicinal plants.

Conflicts of interest: Authors declare no conflict of interest.

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