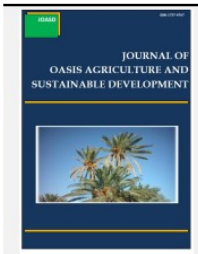

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Phytochemical Screening, Antioxidant and Antimicrobial Activists of *Reamuria vermiculata* leaves

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Abstract

The aim of this study is to determine the phytochemical analyses, antioxidant and antimicrobial activities of *Reamuria vermiculata* leaves extracts. Organic extracts (methanol, hexane, ethyl acetate, petroleum ether) were screened for their biochemical composition as well as antioxidant and antibacterial activities. In fact, Phytochemical screening of *Reamuria vermiculata* leaves showed the presence of flavonoids, coumarins, saponins, tannins, terpenes, and quinones. Biochemical screening showed that the methanol was the richest extract in phenolic and tanins with 179.68 mg GAE/ g and 56.25 mg CE/g, respectively. The analysis of DPPH radical scavenging activity and ABTS showed an important scavenging with ethyl acetate extract with IC50 of 0.0012mg/mL, and 0.0006mg/mL respectively. As well as, the methanol extract exhibited an antimicrobial property against bacterial strains. *Listeria monocytogenes* and *Salmonella typhimurium* were the most sensitive strains with minimum inhibitory concentration values of 0.3125 mg/mL. These results allow us to propose that *Reamuria vermiculata* is an excellent source for bioactive molecules exhibiting interesting biological capacities.

1. INTRODUCTION

In recent years, special attention has been paid to the field of free radical chemistry and reactive oxygen species (ROS) in biology which are producing a medical revolution in health and disease management (Aruoma, 2003). Free radicals cause direct or indirect cellular lesions which lead to pathological modifications (Ashok and Ali, 1999), DNA damage and aging. In order to protect against oxidative stress caused by ROS, the human body has several mechanisms for producing antioxidants (Matés and Sánchez-Jiménez, 1999), which are either produced naturally in situ or supplied from food. These antioxidants, including glutathione, ubiquinol and uric acid, which are produced during the body's normal metabolism, delay or inhibit cell damage especially by their ability to kill free radicals (Shi et al., 1999). Furthermore, due to the fact that the body cannot manufacture some micronutrients which are considered as antioxidants, they are provided in the diet, such as, vitamin E (α-tocopherol), vitamin C (ascorbic acid) and β–carotene (Ausman, 1999). Therefore, there is a current research on natural polyphenolic antioxidants derived from plants to replace synthetic antioxidants. Many scientists and researchers have drawn attention to the bioactive compounds isolated from plant species used in the preparation of folk remedies in the search for sources of natural antioxidants (Stévigny et al., 2005), In this regard, epidemiological studies suggest that long-term consumption of diets rich in plant polyphenols offers protection against the development of cancer, diabetes, osteoporosis, cardiovascular and neurodegenerative diseases (Arts and Hollman, 2005; Graf et al., 2005), Thus, phenolic compounds are the subject of growing scientific interest because of their potential therapeutic benefits on human health. So, the objective of the present study is to determine the phytochemical composition and the antioxidant activity of

different extracts of *Reamuria vermiculata* leaves.

2. MATERIAL AND METHODS

2.1. Plant material

R. vermiculata (Tamaricaceae) plant was collected during the month of March 2015 from the region of Medenine (south of Tunisia, 33°21′17″ North 10°30′19″ East).The plant was taxonomically identified and biochemically evaluated by botanists from arid lands and oasis cropping laboratory (Institut des Régions Arides, Route El-Jorf, 4119 Medenine, Tunisia). The leaves were separated cleaned, dried at room temperature for 15 days and then ground into a fine powder and stored at −20 °C until phytochemical analysis.

2.2. Preparation of extracts

Phenolic compounds were extracted at room temperature using four different solvents, hexane, ethyl acetate and methanol and petroleum ether. One g of each dried organ was grounded and dissolved in 10 mL of each solvent. The mixture was continuously stirred for 6 h in the dark and then filtered through a Whatmann paper (3 mm). The extract obtained was centrifuged for 20 min at 25 °C. Dried extracts were stored at 4 °C in dark until further analysis

2.3 Preliminary phytochemical screening

Preliminary phytochemical screening was carried out with the following methods. The results of the tests were qualitatively expressed as negative (-) or positive (+).

2.3.1.Flavonoids (alkaline reagent test)

A volume of 2 mL of sodium hydroxide 2% was mixed with 5 mg of each extracts to give an intense yellow color. The disappearance of the color after addition of diluted hydrochloride acid indicates the test as positive (Raaman, 2006).

2.3.2 Terpenes (Salkowski's test)

A volume of 2 mL chloroform was mixed with 5 mg of each extract. Then 3 mL concentrated sulphuric acid was added. The formation of a reddish-brown color shows the test as positive (Raaman, 2006).

2.3.3 Alkaloids

Two methods are used to indicate the presence of the alkaloids: Dra-gendorff's test: 2 mL 1% HCl and 2 mL MeOH were homogenized with 5 mg of the extracts along the side of the test tube, then 500uLDragen-dorff's reagent was added to the mixture. The formation of orange or orange-reddish-brown precipitate indicates the presence of alkaloids (UC and NAIR, 2013).

Mayer's test: a drop or two of Mayer's reagent was mixed with 1 mg/mL of the extract. The formation of a white or a creamy pre-cipitate confirms the test as positive (UC and NAIR, 2013).

2.3.4 Phenols and tannins

A volume of 1 mL distilled water was dissolved in 10 mg of each extract and then, few drops of 5% ferric chloride in methanol were added. A bluish-black color shows the test as positive (Banso and Adeyemo, 2006; Raaman, 2006).

2.3.5 Saponins.

5 mg extract was added with 10 mL distilled water. The solution was shaken well for 5 min. The formation of stable foam indicates the presence of saponins (Raaman, 2006).

2.3.6 Terpenes (Salkowski's test)

2 mL chloroform was mixed with5 mg of each extract. Then 3 mL concentrated sulphuric acid was added. The formation of a redish-brown color which confirms the presence of terpenoids (Raaman, 2006).

2.3.7 Sterols

A unit of 5 mg extract with 2 mL H2SO4 was added to 2 mL glacial acetic anhydride. The presence of steroids is indicated by the change of color from violet to blue or green (Edeoga et al., 2005)

2.3.8. Cardiac glycosides (Keller–Kiliani's test)

For the characterization of Cardiac glycosides, the Keller–Kiliani's test has been adopted. A total of 1 mL glacial acetic acid and few drops of 5% ferricchloride solution were mixed with 2.5 mg extract. Further 0.5 mL con-centrated sulfuric acid was added along the side of the test tube. The appearance of a green or blue color shows the presence of cardiac glycosides (Raaman, 2006)

2.4. Determination of total phenolic content

The concentration of total phenolic compounds was determined using Folin–Cicalteu reagent, as adapted by Singleton and Rossi (1965). One hundred µl of the methanol extracts were assayed with 750 µl of 6% (w/v) sodium carbonate and 750 µL of the Folin reagent. After

incubation for 90 min at room temperature in the dark, the absorbance was determined at 765 nm. Three measurements were performed on each sample. The results were expressed as mg of gallic acid equivalents (GAE)/g of dry weight (DW) (mg GAE/g DW).

2.5. Determination of Total Flavonoid Contents (TFC)

The total flavonoids content was measured by the colorimetric assay described by Djeridane et al. (2006). One mL of appropriately diluted samples or standard is supplemented with 1 mL of a fresh solution of aluminum chloride (AlCl3, 2%) After 10 min incubation at room temperature, mixture Absorbance (pink color) was determined at 460 nm and compared to control tube. The total flavonoid content of the different extracts was expressed as mg of catechin equivalents (CE)/ g of dry weight of plant material (mg CE/g DW).

2.6 Determination of Total tannin content (TTC)

In the reaction tube, 2 mL of a freshly prepared solution of vanillin (1% w/v vanillin solution in methanol) were added to 2 mL sulfuric acid (25% v/v sulfuric acid solution in methanol). To each tube 0.5 mL of suitably diluted samples (polyphenol extract corresponding to 1 g of dry plant material) were added. The mixture was incubated in the dark and after exactly 15 min; the absorbance was measured at 500 nm in a spectrophotometer compared to control tube (Oueslati et al., 2012).The Condensed tannins content of the different extracts was expressed as mg of catechin equivalents (CE)/g of dry weight of plant material (mg CE/g DW).

2.7. Antioxidant activity

2.7.1 DPPH essay

The antioxidant activities of leaves extracts were measured in terms of hydrogen donating or radical scavenging ability using the DPPH method as described by Chakraborty and Paulraj (Chakraborty and Paulraj, 2010). The various extracts and ascorbic acid at different concentrations were pipeted in separate test tubes. A unit of 0.5 mL of each sample and standard was added to the same volume DPPH• methanolic solution. After stirring the mixture, the solution is placed in darkness for 30 min at room temperature. Then, the absorbance of the mixture was determined at 520 nm and compared to the control. A mixture of 0.5 mL DPPH• solution and 0.5 mL methanol was taken as a control. Pure methanol was taken as a blank. The DPPH scavenging effects of the samples, in %, were calculated according to the following equation:

DPPH radicals scavenged (%) = [(A0−A1)/A0] × 100,

Where A0 is the absorbance of the control reaction and A1 is the absorbance of the tested extract sample.

2.7.2. ABTS essay

Free radical scavenging activity of plant samples was determined by ABTS radical cation decolorization assay (Chakraborty and Paulraj, 2010).ABTS·+ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), The mixture was stored in the dark at room temperature for 12-16 h. Organic and aqueous extracts samples were dissolved in methanol and in distilled water, respectively, to yield different concentrations. The different concentrations of the extracts and of the ascorbic acid standard were tested. The standard was used for comparison. The measure of the antioxidant activity was realized by adding 100 µl of each standard and sample to 900 µl of diluted ABTS the absorbance was measured at 30 min after the initial mixing at734 nm. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent inhibition of absorbance at 734 nm was calculated using the formula,

ABTS·+ scavenging effect (%) = ((AB–AA)/ AB) ×100 (2),

Where, AB is absorbance of ABTS radical + methanol;

AA is absorbance of ABTS radical + sample extract/standard.

2.8. Antimicrobial activity

Determination of minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations

The MIC of the samples was determined by microdilution on a plate divided into 96 wells, it was defined as the lowest concentration of sample that inhibited the microbial growth after incubation at 37 C for 18–24 h. The MBC and MFC were determined by subculture on blood agar at 37 C for 24 h.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

The phytochemical analysis has shown the presence of some chemical constituents or bioactive compounds from the leaves of *R vermiculata.* These analysis indicated that tannins, flavonoids, saponins, quinones, coumarins, terpenes are found in the methanolic, petroleum ether and ethyl acetate extracts. However, the alkaloids are not detected in all the studied extracts. Only ethyl acetate extract contains cardiotonic glycosides.

3.2. Total polyphenols, flavonoids and condensed tannins contents

Total polyphenols, flavonoids and condensed tannins contents of the different extracts from *Reaumuria vermiculata leaves* were determined.

Total polyphenols, flavonoids and condensed tannins contents of the different extracts from *Reamuria vermiculata* leaves were determined. The results were presented in Table 1. The total polyphenols are significantly affected by the extraction solvent ($p < 0.05$) and the solubility of these compounds in the used solvent. In fact, methanol extract have the highest polyphenol contents (221.75 mg GAE/ g), while, hexane extract present the lowest polyphenols contents (38.32 mg GAE/g). Depending on the used solvents, our findings agree with those of (Trabelsi et al., 2010) which reported that soluble phenolic compounds could be easily extracted from plant tissue using methanol and more effective than less apolar ones, like hexane.

For flavonoids, the highest amount was recorded with the solvents ethyl acetate (126.92mg QE/g) and DCM (80.50 mg QE/g) with significant differences ($p< 0.05$) between the solvents. Considering condensed tannins. The best yields in *R. vermiculata* leaves were obtained in dichloromethane extract (124.75mg CE/g) closely followed by ethanol and acetate respectively (69. 27 mg CE/g; 69.00 mg CE/g). There is no significant difference between ethanol and acetate extracts.

No published study was found in the literature about the leaves of this species. However; a study had been described concerning the polyphenolic content in *R. vermiculata* aerial part collected from Gabes, Tunisia (Karker et al., 2016). It measured that the highest quantity of phenol (117.12 mg gallic acid / g), were obtained in methanol extract. Similarly, the best yields of flavonoid were also recorded in methanol extract (29.9 mg CE/g). Considering total tannins contents, the best yields in *R. Vermiculata* shoots were obtained in dichloromethane extract (27.98 mg CE/g). These amounts are very low compared to our results.

Furthermore, Ksouri et al. (2008) found that the amount of polyphenol in the halophyte plant *Tamarix gallica* leaves in MeOH extract (20.69 mg GAE/g) was very lower compared to the Tunisian halophyte *R. vermiculata.* (221.75 mg GAE/g) of extract. According to (Fratianni et al., 2007), this difference may be explained by different factors such as genetic, environmental including the regions and seasons of the plant collect, the studied part (leaves vs aerial part), the regions and ecological conditions .Previous studies demonstrated that extraction of phenolic compound was affected by the extraction solvent (Ben Yakoub et al., 2018; Elfalleh and BulentKirkan, 2019) and the solubility of these compounds in the used solvent. The variation in phenolic compounds among extracts is mainly due to their different chemical properties which affect widely their solubility in solvents with different polarities (Chan et al., 2009). In another way, (Luo et al., 2002) reported that phenolic compounds, are considerably involved in many plants antioxidant efficiency. Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compound (Krings and Berger, 2001). Thus, the richness of *R. Vermiculata* on phenolic contents allows us to study her potential biological activities.

Table 1. Phenolic content (total polyphenol content, flavonoid and condensed tannin) of different extracts of *Reaumuria vermiculata* leaves.

Extracts	Flavonoids	Tannins	Polyphenols
HXE	41,83±2,12*a*	34,25±0,35*a*	38,32±0,32*a*
EtOAc	126,92±8,88*c*	69,00±3,53*c*	86,82±0,64*d*
MeOH	55,08±7,18*a*	67,50±4,24*c*	221,75±5, 30*e*
PEE	28,00±1,17a	23,50±2,82*b*	14,64±1,92b

3.3. DPPH radical-scavenging activity

The results of the DPPH radical-scavenging assays for the tested extracts are presented in Table 2. The acetate extract showed the highest scavenging ability comparate to ascorbic acid (IC50= 0.0012 mg/mL) followed by MeOH and

EtOAc extract fractions, respectively. The DPPH radical scavenging activity of the leaves extracts can be correlated to the contents of a flavonoids, phenol and tannin. No published study was found about the DPPH radical scavenging activity from *R vermiculata* leaves extracts. Another study was concentrated on the leaves of medicinal halophyte Tamarix *gallica.* Ksouri et al (2008) studied the antioxidant capacity of the MeOH extract (IC50= 0.007mg/mL) using DPPH technique. Its IC_{50} was very lower compared with our finding of the MeOH extract (IC50=0.004 mg/mL).

3.3.2. ABTS radical scavenging activity

The antioxidant activity of *R. vermiculata* leaves extracts was assessed in vitro using ABTS radical scavenging assay. It is seen in Table 2 that the ethyl acetate extracts had strong effects of ABTS scavenging free radical with an average IC_{50} value of 0.0006 mg/mL. While the hexane one (IC_{50}=0.045 mg/mL) was poorly extract. These results showed that varying solvent polarities differ significantly in their extraction capacity of antioxidant compounds, and therefore, in their antioxidant activities. The high amount of TPC, TFC, and TTC can explain this strong activity. No published study was found in the literature about the ABTS radical scavenging activity from *R vermiculata* leaves.

Table 2. IC_{50} (mg/mL) values of the extracts for free radicals scavenging activity by DPPH, and ABTS radicals

	DPPH	ABTS
HXE	0,039±0,016^{c}	0,045±0,001^{e}
EtOAc	0,001±0,001^{a}	0,001±0,000^{a}
MeOH	0,004±0,001^{ab}	0,006±0,000^{b}
PEE	0,007±0,001^{b}	0,039±0,006^{b}
Ascorbic acid	0,003± 0,000^{ab}	0,015± 0,001^{c}

3.4. Antibacterial essays

The different *Reaumuria vermiculata* extracts showed various degrees of inhibition, evaluated as MIC and MBC against the tested strains. The results were compared to gentamicin used as positive control for bacteria (Table 3). The methanol extract had a significant inhibitory effect on Gram+ and Gram− bacterial strains, this effect was highlighted by the measure of MIC which reached its minimum value against *Listeria monocytogenes* and *Salmonella typhimurium* (MIC= 0.039 mg/mL). However, *Escherichia. Coli* and *staphylococcus aureus* were more resistant with MIC of 2.5 mg/ml and 1.25 mg/ml, respectively.

Table 3. Antibacterial activity of roots extracts from *R. vermiculata (*mg/mL).

Strains		MEE	PEE	HXX	EtOAc
E. coli	**MIC**	**2.5**	>5	**5**	**2.5**
	MBC	**>5**	**>5**	**>5**	**>5**
Listeria monocytogenes	**MIC**	**0.039**	>5	>5	>5
	MBC	**5**	>5	>5	>5
Salmonella typhimurium	**MIC**	**5**	>5	>5	>5
	MBC	**>5**	>5	>5	>5
S. aureus	**MIC**	**1.25**	>5	>5	>5
	MBC	**1.25**	>5	>5	>5

MIC*: Minimum inhibitory concentration;* ***MBC****: Minimum bactericidal concentration*

4. CONCLUSION

On basis of the results obtained in the present study, it was concluded that the biochemical screening of the leaves show that the highest total polyphenolic content values were recorded in the methanol. The highest flavonoids amount was registered with the solvent ethyl acetate with considering condensed tannins. .The acetate extract showed the highest antioxidant activities. The DPPH and ABTS scavenging activities are positively correlated with the content of phenol and flavonoid compounds. These results enhance the therapeutic virtues of the *R.vermiculata* leaves used in traditional medicines in Tunisia as a source of antioxidant bio-compound, and suggests its use as valuable source of natural bioactive molecules

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