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Preliminary phytochemical analysis and antibacterial potential of organic extracts from aerial parts of *Retama monosperma*

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ABSTRACT

The present study was designed to evaluate the phytochemical profile and potential antibacterial activity of *R. monosperma* aerial parts extracts. The aerial parts extracts showed the presence of alkaloids, terpenoids, tannins, saponins, flavonoids and coumarines. In addition, total phenols, flavonoids as well as tannins contents were determined in four organic extracts from stems, flowers and seeds of *R. monosperma* and used to assess their contribution to a potential antibacterial effect. Stems and seeds methanol extracts exhibited high total phenols content (495.75 mg GAE/g and 195.69 mg GAE/g) respectively. In contrast, the methanol extract of the flowers showed lower total phenolic content (146.77 mg GAE/g) but higher tannins content (381.92 mg CAE/g). When compared to total phenols and tannins contents, the aerials parts of *R. monosperma* extracts showed low contents of total flavonoids (33.37 QE/g to 65.88 QE/g). The *in vitro* antibacterial activity of *R. monosperma* aerials parts extracts at a concentration of 500 mg/ml was investigated against various strains of bacteria by disc diffusion method. The ethyl acetate extracts of the stems and the flowers demonstrated a very significant activity (Ø > 14mm) on *Salmonella sp* meanwhile, hexane and dichloromethane extracts from seeds exhibited very significant antibacterial effect against *Bacillus sp* and *Escherichia coli*.

1. Introduction

In Morocco, Medicinal plants have been used in traditional medicine at relatively lower expenses than in modern medications. The curative properties of medicinal plants are mainly due to the presence of various secondary metabolites such as alkaloids, flavonoids, terpenoids, glycosides saponins, tannins *etc*.

Retama monosperma is classified in the *Genisteae* tribe of the *Fabaceae* family. It is grows in the north of Africa and on the Iberian Peninsula. *R. monosperma* is one of the botanical species that grows spontaneously and abundantly in the natural forests and meadows of the Atlantic coast of El Jadida (Morocco). This plant is of ecological interest in dune stabilization and soil fixation of semiarid and arid ecosystems. In Morocco, *R. monosperma* has long been regarded as ornamental plant and also as an effective medicinal plant [1, 2].

Previous phytochemical investigations of the *Retama* genus have resulted in the identification of alkaloids [3, 4], terpenoids [5-7], flavonoids [8, 9], and fatty acids [4, 10]. The antimicrobial [6, 7, 11-13], cytotoxic [4, 9], analgesic [8] and antioxidant activities [12-14] of *Retama* genus are well documented in the literature.

Many plants have been used for treating disease caused by microorganisms such as cholera, diarrhea, dysentery *etc*..[15]. In addition, a large economy is invested in the imports of drugs especially antibiotics from different parts of the world. Thus, antibacterial activity of local medicinal plants should be studied to provide alternative antibacterial treatments. Therefore, the aim of the present study was to determine total polyphenols, flavonoids and tannins contents in *R. monosperma (*L.) Boiss. and evaluate its antibacterial activities against some human pathogenic bacteria.

2. Materials and methods

2.1. *Plant material*

The plant material was collected from Haouzia (El Jadida city, Atlantic coast, Morocco) during the spring 2014 (February for flowers and stems, April for seeds). Dr. M. Fennane from Scientific Institute of Rabat, Morocco, authenticated the plant. A voucher specimen (77816 RAB) was deposited in the Herbarium of the Institute.

2.2. *Plant extraction*

The plant samples were air-dried for several weeks. Powdered seeds, stems and flowers were extracted three times by maceration with methanol. The resultant extracts were concentrated under reduced pressure. The methanol crude extracts were solubilized in water and extracted successively with equal volumes of three organic solvents of increasing polarity (hexane, dichloromethane and ethyl acetate) to give three fractions. Each fraction was evaporated to dryness under vacuum and stored at +4°C until tested after determining the weight and the yield. Prior the antibacterial experiment, a concentration of 500 mg/ml was prepared by reconstituting the crude extracts in absolute methanol.

2.3. *Phytochemical screening*

The fresh methanolic crude extracts were qualitatively screened for the following constituents: flavonoids, coumarines, tannins, alkaloids, terpenes, anthraquinones and saponins according to Harborne [16]. The qualitative results have been rated from (+) for faint to (++++) for dense turbidity or colour.

2.4. *Phenolic compound analysis*

2.4.1. Total phenolic content

The polyphenol contents of the extracts were determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method [17]. A calibration curve was prepared with gallic acid and the results were expressed as mg gallic acid equivalent (GAE)/g extract. Data presented are average of three measurements.

2.4.2. Total flavonoid content

Total flavonoids were measured using a colorimetric assay developed by Dewanto et *al*. [18]. An aliquot of diluted extract or standard solution of quercetin was added to 75 µl of $NaNO_2$ solution (7%), and mixed for 6 min, before adding 0.15 ml $AlCl_3$ (10%). After 5 min, 0.5 ml of 1 M NaOH solution was added. The final volume was adjusted to 2.5 ml, thoroughly mixed, and the absorbance of the mixture was determined at 510 nm. Total flavonoids were expressed as mg quercetin equivalent (QE)/g extract, through the calibration curve of quercetin (0–400 µg/ml range). All extracts were analysed in three replications.

2.4.3. Total tannin content

Condensed tannins were measured using the modified vanillin assay described by Sun et *al*. [19]. Three millilitres of 4% methanol vanillin solution and 1.5 ml of concentrated H_2SO_4 were added to 50 µl of suitably diluted sample. The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm against methanol as a blank. The amount of total condensed tannins was expressed as mg catechin equivalent (CAE)/g extract. All samples were analysed in three replications.

2.5. *Bacterial cultures*

The antibacterial screening was conducted against four Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus sp* (CIP 104717)*, Bacillus cereus* (ATCC 33019), *Listeria ivanovii* (ATCC 19119), and three Gram negative bacteria: *Escherichia coli* (*CIP 54127*)*, Citrobacter freundii* (ATCC 8090) and *Salmonella sp*. All the microorganisms were obtained from Pasteur Institute (Casablanca, Morocco). They were maintained by periodical subcultures and preserved at +4°C prior to use.

2.6. Preparation of inoculums

The tested bacteria were sub-cultured in nutrient broth for about 18 h at 37°C to reach the exponential phase and then adjusted to 0.5 McFarland's standard, using sterile normal saline, to get bacterial density equivalent to approximately 1.0×10^8 CFU/ml, and directly employed in the antibacterial testing.

2.7. Disc diffusion method

The antibacterial activity of *R. monosperma* extracts was determined using a modified Kirby-Bauer disc diffusion method as reported by Kowti et *al*. [20], with some modifications; In aseptic conditions, 20 ml of warm Mueller Hinton agar, were poured on sterile disposable plates and left at room temperature to solidify,

The plates were then turned upside down and kept in the refrigerator for approximately a half an hour. One hundred µl of the bacterial suspensions (previously adjusted) were swapped onto the Mueller Hinton plates, using sterile cotton swaps. Sterile blank discs of 6 mm were previously prepared from Whatman No.1 filter paper (Sigma-Aldrich). They were impregnated with 20 µl of three concentrations of each extract (500, 250 and 125 mg of extract/ml methanol). Saturated discs were placed onto inoculated plates. The plates were allowed to stand for one hour at room temperature, and then incubated at 37°C for 24 hrs. Tetracycline (30 µg/disc) and Penicillin G (30 IU/disc), were used as positive controls. Solvent control was prepared with discs saturated with methanol, while virgin sterile disc were used as blank control. The susceptibility of the tested bacteria to the extracts was evaluated by measuring the diameters of the zones of growth inhibition in millimeters (mm) using a transparent ruler. All the experiments were performed in triplicate and the results were expressed as mean value ± standard deviation.

The antibacterial efficiency of the extracts was classified as follows:
Ø ≤ 8mm: No significant activity.
8 < Ø ≤ 12mm: Moderate activity.
12< Ø ≤ 14mm: Significant activity.
Ø > 14mm: Very significant activity.

2.8. Statistical analysis
All data sets were expressed as Mean ± SD. Data was also statistically analysed using one-way ANOVA (analysis of variance) followed by Bonferroni post hoc test. The differences were considered significant at $P<0.05$.

3. Results and discussion

3.1. *Extraction and solvent fractionation*
The yields of the crude extracts of *R. monosperma* seeds, stems, flowers and theirs respective fractions (*n*-hexane, dichloromethane, ethyl acetate) are presented in **Table 1**. The maximum yield was obtained for the crude extracts of flowers (24.14%) followed by stems (20.13%). The ethyl acetate fractions of stems and flowers as well as the dichloromethane fractions of seeds and flowers are negligible.

Table 1. Percentage yields of *R. monosperma* seeds, flowers and stems methanol crude extracts and various fractions.

R. monosperma	**Methanol**	***n*-hexane**	**Dichloromethane**	**Ethyl acetate**
stems	20.13	4.86	1.65	0.34
seeds	18.04	2.57	0.53	2.27
flowers	24.14	3.82	0.15	0.33

3.2. *Phytochemical screening*
The phytochemical constituents of *R. monosperma* seeds, flowers and stems methanol extracts (**Table 2**) proved to be rich in alkaloids, tannins and flavonoids with no anthraquinones detected neither quinones. Flowers were poor in coumarines and saponosides but the stems were rich in terpenoids. These results are in agreement with previous studies on *Retama* genus [3, 11, 21-22].

3.3. *Phenolic contents analysis*
The phenolic contents (total polyphenols, flavonoids and tannins) of the different parts of *R. monosperma* are reported in **Table 3.** The highest total polyphenols contents was recorded in the methanol extract of the stems (495.75 mg GAE/g dry extract) while the methanol extract of the flowers showed a higher tannins content (381.92 mg CAE/g dry extract) than the other extracts. If compared to *R. monosperma* growing in Algeria [14], the Moroccan species has a similar content of total phenols but a high content of tannins. Flavonoids content of the extracts of *R. monosperma* ranged from 33.37 QE/g (in ethyl acetate extract of stems) to 65.88 QE/g (in dichloromethane fraction of flowers). It was higher than that reported in *R. monosperma* growing in Algeria [14], but similar to that of *R. raetam* flowers of Tunisia [13].

Table 2. Phytochemical screening of the methanol crude extracts of aerials parts of *R. monosperma*

Phytochemical Compounds	Flowers	Seeds	Stems
Alkaloids	++	+++	+++
Tannins	+++	++	+++
Flavonoids	+++	+++	+++
Anthraquinones	-	-	-
Quinones	-	-	-
Saponosides	+	-	+
Terpenoids	++	-	++
Coumarines	+	+	-

3.4. *Antibacterial activity*

In this study crude methanol, hexane, dichloromethane and ethyl acetate extracts from aerials parts of *R. monosperma* were tested for their antibacterial activity against seven human bacterial pathogens. At concentrations of 250 mg/ml and 125 mg / ml, all the studied extracts were inactive against all tested strains (data not shown). The highest antibacterial activity was observed at a concentration of 500 mg/ml in seeds followed by flowers, while the stems showed relatively lower activity (**Table 4**).

Seeds: The hexane, ethyl acetate and dichloromethane extracts were found to be effective in inhibiting the growth of all tested bacteria with diameters of zones of inhibition ranging from 8 to 15 mm. The hexane and the dichloromethane extracts showed excellent growth inhibitory activities against *Bacillus sp* and *Escherichia coli* respectively (15mm). In addition, the methanol extract of the seeds proved to have an antibacterial activity against four selected pathogens, *Bacillus sp, Bacillus cereus, Listeria ivanovii* and *Escherichia coli;* while it showed no effect on *Staphylococcus aureus, Citrobacter freundii* and *Salmonella sp* growth (**Table 4**).

Flowers: The dichloromethane extract of the flowers exhibited a bacteriostatic activity against all tested strains with the maximum inhibition zone on *Listeria ivanovii* (14 mm). In contrast, hexane extract showed an inhibitory effect on only *Bacillus cereus, Bacillus sp,* and *Salmonella sp* with an inhibition zones diameters of 12 mm, 13 mm and 12 mm respectively (**Table 4**). The ethyl acetate extract exhibited an inhibitory growth activity against four bacteria within the maximum zone of inhibition on *Citrobacter freundii* (14 mm) and *Salmonella sp* (15 mm). *Staphylococcus aureus, Citrobacter freundii* and *Salmonella sp* were resistant to the methanol extract while a moderate effect was observed against *Bacillus sp* and *Listeria ivanovii* (12 mm and 10.5 mm respectively).

Stems: The highest antibacterial activity was observed with the ethyl acetate extract witch display an inhibitory effect against *Listeria. ivanovii* (13 mm) and *Salmonella sp* (15.33 mm) respectively, whereas *Bacillus cereus*, *Citrobacter freundii* and *Staphylococcus aureus* were moderately inhibited (**Table 4**). The methanol extract showed comparatively a week antibacterial activity; it developed a moderate or low effect on only three bacteria *Bacillus sp* (10 mm), *Bacillus cereus* and *Escherichia coli* (9 mm). *Staphylococcus aureus, Citrobacter freundii* and *Escherichia coli* were resistant to the hexane extract whereas *Bacillus sp* and *Escherichia coli* were resistant to the ethyl acetate extract. It is worthy to note, that both standards (Penicillin G and Tetracycline) exhibited a broad-spectrum antibacterial activity against all Gram positive and Gram negative bacteria tested in a dose-dependent manner, albeit to varying extent. Otherwise, as expected negative control (solvent) exhibited no significant effect (data not shown).

Table 3. Total polyphenols, flavonoids and tannins contents of *R. monosperma* aerial parts extracts

Extracts	Stems			Flowers			Seeds		
	Polyphenols mg/ GAE/g	**Flavonoids mg/QE/g**	**Tannins mg/ CAE/g**	**Polyphenols mg/ GAE/g**	**Flavonoids mg/QE/g**	**Tannins mg/ CAE/g**	**Polyphenols mg/ GAE/g**	**Flavonoids mg/QE/g**	**Tannins mg/ CAE/g**
Methanol	495.75±0.00	62.18±0.06	336.9±0.02	146.77±0.03	49.63±0.03	381.92±0.02	195.69±0.00	46.53±0.05	227.17±0
Hexane	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dichloromethane	328.76±0.00	61.42±0.03	285.87±0.06	74.31±0.01	65.88±0.16	220.47±0.04	163.07±0.04	44.93±0.23	199.93±0.17
Ethyl acetate	157.64±0.04	33.37±0.02	272.57±0.01	160.37±0.01	55.83±0.04	65±0.01	112.19±0.03	39.63±0.04	167.16±0.00

mg GAE/g: Milligram-gallic acid equivalent per gram of extract
mg QE/g : Milligram-quercetin equivalent per gram of extract
mg CAE/g Milligram-catechin equivalent per gram of extract
ND: Not determined

Table 4. Antibacterial activity of the extracts of stems, flowers and seeds of *R. monosperma*

	Extracts	**Diameter of the zone of inhibition (mm)**						
		Gram positive				**Gram négative**		
		Bacillus sp	***Bacillus cereus***	***Listeria ivanovii***	***Staphylococcus aureus***	***Citrobacter freundii***	***Escherichia coli***	***Salmonella sp***
Stems	**Methanol**	10 ±0	9 ±1	NE	NE	NE	9±1	NE
	Hexane	10±0.5	10±0	13±2	NE	NE	NE	8±1
	Dichloromethane	10±1	NE	10±2	NE	10±2	10±2	NE
	Ethyl acetate	NE	11±1	13 ±2	10±1	10±3	NE	15.3±0.5
Flowers	**Methanol**	12±3	8 ±2	10.5±0.5	NE	NE	9±1	NE
	Hexane	13±2	12±1	NE	NE	NE	NE	12±1
	Dichloromethane	10±1	10±1	14±0	11±2	10±2	10±2	12±1
	Ethyl acetate	NE	11	11.33 ±1	NE	14±1	NE	15 ±0
Seeds	**Methanol**	13 ±2	10 ±1	9 ±1	NE	NE	9.3±0.5	NE
	Hexane	15±1	10±1	11±1	10±1	10±2	10±2	8±1
	Dichloromethane	12±2	10±2	11±3	10±2	11±3	15±1	10±1
	Ethyl acetate	10±1	11±1	12±2	10±2	10±1	10±1	13±2
	Tetracycline	13±2	12±1	20±4	15±2	9±3	10±2	10±1
	Penicillin G	10±2	12±3	10±2	11±2	11±2	12±2	8±3

NE: No effect

Our results show that the antibacterial potential varies according to the nature of the solvent and the part of the plant used. Overall, the seeds have the best potential followed by flowers and stems. These results are in agreement with previous studies on *Retama* genus, and which have reported the antibacterial effect of various extracts especially from neighboring species *R. raetam* against some human bacterial pathogens [6, 12, 13]. The antibacterial potential of *R. monosperma* aerial parts extracts against all tested strains is clearly demonstrated. However, these results expressed qualitatively by the measurement of the zone of inhibition, must be expressed quantitatively by determining the MICs (Minimum Inhibitory Concentrations).

Based on a literature survey on *Retama* genus, there are some few studies dealing with the phytochemistry [3, 10], the pharmacology [14] and the antifungal properties of *R. monosperma* [11], but there is no report on its antibacterial activity. Thus, the present study shows for the first time the antibacterial potential of *R. monosperma* extracts.

The antibacterial activity of *R. monosperma* aerial parts extracts can be related to their contents of several bioactive components (**Table 2-3**) which generate synergistic effects. In fact, the genus *Retama* is known for the presence of variety of compounds which have been found effective antimicrobial substances against a wide array of microorganisms *in vitro*. Previous investigations led to the identification and/or isolation of alkaloids [3, 4, 23], flavonoids [8], flavonoids glucosides [8, 9, 24, 25], phenolic compounds [8] and terpenoids [5-7].

Moreover, previous works on *R. raetam* indicated that two flavonoids, licoflavone and derrone, isolated from ethyl acetate extract of flowers [9] were active against *Pseudomonas aeruginosa* and *Escherichia coli.* In ours previous study, we have repoted that the dichloromethane extract of aerial parts of *R. monosperma* were also rich in quinolizidine alkaloids [3, 11], which showed significant activity on *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [26].

Conclusions

To the best of our knowledge, we herein report for the first time the antibacterial profile of *R. monosperma* extracts growing in Morocco. In this study, it can be concluded that from the overall results of the antibacterial assays, *R. monosperma* extracts form a good basis for further investigation in the potential discovery of new natural bioactive compounds. By isolating and identifying these bioactive compounds, new drugs can be formulated naturally to treat various infectious diseases.

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