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Antifungal activity of the alkaloids extracts from aerial parts of *Retama monosperma*.

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ABSTRACT

We have investigated the antifungal activity of alkaloids extracts from aerial parts of *Retama monosperma* using the disc diffusion method. The alkaloids extracts from seeds and flowers were inactive against all microorganisms tested (*Candida albicans, Candida tropicalis* and *Aspergillus niger*). Both extracts obtained from the stems and leaves were found to be active in a dose dependent way, albeit to varying extent. The maximum degree of antifungal activity was observed in leaves extract followed by stems extract. The most abundant compounds in alkaloids extracts of leaves and stems, namely sparteine, ammodendrine and anagyrine might be responsible for their antifungal activities. The high percentages of cytisine and its derivatives in alkaloids extract of seeds of *R. monosperma* might be responsible for its inactivity.

Keywords: Antifungal activity, *Retama monosperma*, Fabaceae, quinolizidine alkaloids,

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INTRODUCTION

Herbal drugs play an important role in health care programs worldwide, and there is a resurgence of interest in herbal medicines for treatment of various ailments [1]. Plants generally produce several secondary metabolites like essential oil, phenols, flavonoids, glycosides, quinones, tannins, alkaloids, saponins, and sterols which are important sources of biocides and many other pharmaceutical drugs [2-4].

Retama genus belongs to the family of the Fabaceae, tribe Genistae and includes three species (*R. monosperma, R. raetam* and *R. sphaerocarpa*) distributed in the Mediterranean, North Africa and the Canary islands [5-7].

In Moroccan folk medicine, *Retama monosperma* (L.) Boiss. and *Retama raetam* (Forsk.) Webb. locally named as "*Rtem*" are used by traditional healers as an emetic, purgative, vermifuge, healing, vulnerary, sedative [6], anthelmintic, antiseptic [7] and antidiabetic [8,9]. *Retama monosperma* (L.) Boiss. is a spontaneous plant that colonizes dune sands. The plant flowers from January to Mars and it is known as well as *R. raetam* for its ornamental flowers and its important ecological roles in dune stabilization and soil fixation [10]. In Morocco, *R. monosperma* is located in the valleys, sandy regions and in the internal areas of the Great Atlas, where the climate is semi-arid [7].

Previous pharmacological studies on the genus have revealed its various medicinal properties: analgesic [11], antibacterial [12,13] , antifungal, antiviral [14], cytotoxic [12,15-17], hypoglycemic, diuretic [18,19], anti-hypertensive [20], antioxidant [11,13,21] and antileukemic effects [22] .

Retama species have been reported to contain flavonoids [11,12,23], essential oils [21,24] and quinolizidine alkaloids [25-27]. Recently, the anticorrosive properties of the alkaloid extract of seeds of *Retama monosperma* have been also reported [28].

To the best of our knowledge, no systematic investigation of the antifungal activity of alkaloids of *R. monosperma* growth in Morocco has been studied hitherto. The present paper deals with the antifungal activities of alkaloids extracts of aerial parts of *R. monosperma.*

MATERIALS AND METHODS

Plant Material

The plant material was collected from Haouzia (El Jadida city, Atlantic coast, Morocco) during spring (April) 2013. Dr. M. Fennane from Scientific Institute of Rabat, Morocco, authenticated the plant. A voucher specimen has been kept in the Herbarium of Institute under the code Ref.77816 RAB.

Extraction of alkaloids

Retama monosperma (L.) Boiss. was collected in the coastal dunes of Haouzia (El Jadida,Western Morocco) and the alkaloids extracts was obtained according to a previously described experimental procedure [27]. 100 g of each part of *Retama monosperma* (L.) Boiss. (stems, leaves, flowers and seeds) dried and finely crushed were extracted with absolute methanol three times at room temperature. After filtration, the methanolic extracts were combined and concentrated to dryness under reduced pressure. The remaining residue was acidified by 5% hydrochloric acid solution, filtered and the acid aqueous solution was then basified with 25% ammonium hydroxide and extracted with dichloromethane. The organic phase was filtered, dried over anhydrous sodium sulfate, filtered again and finally concentrated in vacuo. The acid-base purification procedure was repeated three times to give a dark brown semi-solid extracts of alkaloids. The alkaloids extracts of *Retama monosperma* parts (seeds, leaves and stems) was analysed by gas phase chromatography–mass spectrometry (GC–MS) technique as described in our previous work [27]. The obtained constituent percentages of the *Retama monosperma* (L.) Boiss. alkaloids extracts are summarized in Table 1.

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Table 1: Chemical composition of *Retama monosperma* alkaloids extracts [27]

alkaloids	Stems (%)	Leaves (%)	Seeds (%)
Sparteine	28.87	27.93	trace
Dehydrosparteine	9.40	0.7	-
β-Isosparteine	-	12.62	-
Ammodendrine	24.36	10	-
N-Methylcytisine	7.93	4.89	13.11
Dehydrocytisine	-	-	9.37
Cytisine	3.23	6.02	77.58
17-oxosparteine	1.61	5.45	-
Isolupanine	5.25	7.74	-
5.6-Dehydrolupanine	7.05	5.73	trace
11.12-Dehydrolupanine	trace	-	-
Anagyrine	12.33	18.93	-
Thermopsine	-	-	trace

Microorganisms and media

In this study, three fungal species obtained from the Collection of Pasteur Institute (Casablanca, Morocco) were used as the antifungal test strains; two yeasts*: Candida albicans* CIP4872 and *Candida tropicalis* R2 CIP1275. 81(an Amphotericin B and Nystatin resistant strain) and one filamentous fungus (*Aspergillus niger* CIP1431.83). All the microorganisms were maintained by subculturing periodically and preserved at 4°C prior to use. For inocula preparation, fungi were incubated for 3 days in Potato Dextrose Agar (PDA).

Agar- disk diffusion method

The disc diffusion test was applied for the evaluation of the susceptibility of microorganisms to the alkaloids extracts [29]. The yeasts cultures in the exponential phase of growth or fungal spore solution were spread on PDA plates in order to give a population of approximately 10^8 CFU/plate.

A sterilized disc (6.0 mm in diameter) of Whatman filter paper N°. 1 were impregnated separately with 20 µl of five concentrations of each extract (500 to 31.25 µg of extract/ml DMSO), and then were placed on the surface of the agar plate.

The plates were kept at 4°C for 2 h and then incubated for 24h (yeasts) or 3 days (filamentous fungi) at 37°C under aerobic conditions and the diameter of the inhibition zone around each disc was then measured and recorded. Nystatin (30 IU/disc) and Fluconazole (30µg/disc) were used as positive control. Negative control was set up with equivalent quantities of solvents used in the extraction process and DMSO. Each test was carried out in triplicate and the results (mm of zone of inhibition) were expressed as mean value ± standard deviation.

The antifungal results of the extracts were classified as follows:

Ø ≤ 8mm: Non-significant antifungal activity.
8 < Ø ≤ 12mm: Moderate antifungal activity.
12< Ø ≤ 14mm: Significant antifungal activity.
Ø > 14mm: Very significant antifungal activity.

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 $P<0.05$.

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RESULTS AND DISCUSSIONS

The alkaloids extracts of *Retama monosperma* were tested for antifungal activity against three human fungal pathogens by using five concentrations (500, 250, 125, 62.5 and 31.25 µg/ml). The seeds and flowers extracts have no antifungal effect, whereas both extracts obtained from the stems and leaves were found to be active in a dose dependent way, albeit to varying extent (Table 2). The maximum degree of antifungal activity was observed in leaves extract followed by stems extract.

Table 2: Antifungal activity of alkaloids extracts from aerial parts of *R. monosperma*

		Diameter[a] of inhibition zone (mm)		
Alkaloids extracts	(µg/ml)	*C. albicans*	*C. tropicalis*	*A. niger*
Stems	500	16.66 ± 2.22	14.33 ± 2.44	9.33 ± 0.44
	250	11.33 ± 1.11	11.05 ± 1.33	8.05 ± 0.17
	125	10.10 ± 1.77	9.66 ± 0.44	8.10 ± 0.88
	62.5	9.60 ± 0.44	NE[b]	NE
	31.25	7.10 ± 0.15	NE	NE
Leaves	500	18.66 ± 1.77	20.66 ± 2.88	8.66 ± 0.34
	250	13.33 ± 0.88	11.66 ± 2.22	7.66 ± 0.41
	125	9.66 ± 0.44	9.33 ± 0.44	7.01 ± 1.33
	62.5	NE	NE	NE
	31.25	NE	NE	NE
flowers	500	NE	NE	NE
	250	NE	NE	NE
	125	NE	NE	NE
	62.5	NE	NE	NE
	31.25	NE	NE	NE
Seeds	500	NE	NE	NE
	250	NE	NE	NE
	125	NE	NE	NE
	62.5	NE	NE	NE
	31.25	NE	NE	NE
Nystatin	30IU/disc	25.10 ± 1.11	NE	14.03 ± 1.01
Fluconazole	30µg/disc	31 ±1.16	NE	8 ±2,01

a : Includes diameter of disc (6 mm); b : No effect

In the highest concentration (500 µg/ml), the alkaloids extracts from leaves and stems presented a very significant antifungal effect against *Candida albicans* (18.66 and 16.66 mm respectively) and *Candida tropicalis* (20.66 and 14.33mm respectively). The alkaloids extract of leaves was more active than stems extract, in particular against *Candida tropicalis.* In low concentrations (250 and 125 µg/ml), the affects exerted by extracts of stems and leaves against *Candida* species (Table 2) are moderates and almost similar except for *C. albicans* which is most inhibited by leaves than stems extract (13.33 and 11.33 mm respectively).

A. niger is also moderately inhibited by stems and leaves extracts in the highest concentration (500 µg/ml) with the maximum zone of inhibition (9.33 and 8.66 mm respectively). *Candida* species and *A. niger* are resistant to alkaloids extracts of seeds and flowers of *R. monosperma.*

It is to note, that Nystatin and Fluconazole, used as standard, inhibited only *C. albicans* and *A. niger* while negative control (solvents) exhibited no significant effect (data not shown).

Immunocompromised patients, such as HIV-infected individuals, transplant recipients and cancer patients are especially vulnerable and die mainly due to opportunistic invasive fungal infections (IFIs) [30]. The most common causative agents of these infections are *Candida* spp., *Aspergillus* species, and *Cryptococcus neoformans* [31]. Amphotericin B and fluconazole are the drugs of choice for treatment of cryptococcosis. However, some recent isolates have shown resistance to fluconazole. In addition, amphotericin B has high toxicity and therefore its use should be limited [32]. Polyenes, azoles and echinocandins are now the main classes of antifungal drugs available to control these infections, but with the changing spectrum of pathogens

and their increasing resistance to these antifungal agents, together with possible side effects produced by current therapies, the development of new antifungal scaffolds is critical [30].

In the present investigation, *in vitro* antifungal efficacy of the crude alkaloids extracts of *R. monosperma* was quantitatively assessed on the basis of zone of inhibition.

The antifungal potential of alkaloids extracts of stems and leaves against all fungi tested is clearly demonstrated (Table 2). Alkaloids from seeds and flowers have no antifungal effect. The alkaloids extract of leaves shows highest activity against both *Candida* species followed by the stems extract. Based on the previous literature on *Retama* genus, there are some recent studies on phytochemistry and pharmacology on *R. monosperma* [16,17,22]., but there is no report on antifungal properties of alkaloids extracts of different parts of this plant. Thus, the present study shows the presence of antifungal activity in *R. monosperma* for the first time.

The presence of antifungal activity in a particular part of *R. monosperma* may be due to the content and/or to the presence of one or more bioactive compounds in crude alkaloids extracts of leaves and stems (Table 1).

In our previous study [27], we reported the chemical composition of quinolizidine alkaloids, as well as their distribution in the stems, leaves and seeds of *Retama monosperma* (L.) Boiss. growing in Morocco (Table 1).

In addition, we reported that chemical profile of the alkaloids extract of stems was qualitatively very similar to that of leaves. Thus, ten alkaloids were identified in leaves: sparteine was the main component (27.93%) followed by anagyrine (18.93%) and β-isosparteine (12.62%). In stems, sparteine was also the major component (28.87%), associated with ammodendrine (24.36%) and anagyrine (12.33%).

Additionally, our previous GC-MS analysis demonstrated that the alkaloids extract of *R. monosperma* seeds contained cytisine as the main component, followed by methylcytisine (13.11%) and dehydrocytisine (9.37%) [27].

A number of biological and toxicological activities of quinolizidine alkaloids such as hypoglycemic, diuretic [18,19,33], antibacterial, antifungal [3,34,35], hypotensive, antiarrhythmic, respiratory stimulant and depressant, antipyretic, hallucinogenic properties have been reported [25,36].

In a previous study of Wink [37], sparteine was reported to have antimicrobial activity against bacteria and phytopathogenic fungui. In addition, various biological effects of anagyrine such as nematocidal and acetylcholinesterase inhibitory property as well as teratogenic effect have been reported in the literature [36,38,39].

Moreover, in a study on *Genista vuralii* [40], it was reported that anagyrine might be responsible for the antifungal effect of the alkaloids extract of *G. vuralii* which has been found to have significant antifungal activity against *Candida krusei*.

Although no antifungal activity for sparteine and anagyrine has been reported to date, these alkaloids might be responsible for antifungal activity of the alkaloids extracts of stems and leaves which have been found herein to have very significant antifungal activity against *C. albicans* and *C. tropicalis.*

Pérez-Laínez et *al*. [34] investigated bactericidal and fungicidal activities of the alkaloids extract obtained from *Calia secundiflora*, they reported that cytisine (the most abundant compound), lupinine, anagyrine, sparteine, N-methylcytisine, 5,6-dehydrolupanine and lupanine were identified in the extract of seeds. The authors demonstrated also that the crude extract of *C. secundiflora* was potent on phytopathogenic fungi (*Alternaria solani, Fusarium oxysporum and Monilia fructicola*) while in contrast, cytisine showed the opposite effect. As it was reported in Table 1, cytisine is the predominant compound in the extract of seeds followed by N-methylcytisine and dehydrocytisine; that explains probably the inefficiency of the alkaloids extract of seeds of *R. monosperma*.

Moreover, according to El-Shazly et *al*. [36]. the flowers of *R. raetam* native to Egypt as well as *R. sphaerocarpa* of Portugal have a high percentage of cytisine (54.25 and 24,16 % respectively); This high level of cytisine can probably help to explain the inactivity of the flowers extract of *R. monosperma* of Morocco.

In a study by Yan et *al*. [35], the antimicrobial activities of total alkaloids from *Sophora flavescens*, and its monomer matrine, oxymatrine, sophoridine, sophocarpine, and cytisine, were tested against 11 plant pathogenic fungi and 3 bacteria using *in vitro* methods. They found that only the total alkaloids to *Phytophthora drechsleri* and *Colletotrichum gloeosporioides, and* sophoridine *to P. drechsleri* had relatively high activities. Recently, it has been reported that *R. raetam* extracts possessed an antifungal activity [3, 12, 13]. In addition, alkaloids extract of *R. raetam* leaves have proved to be effective against four fungi (*Trichoderma sp, Sclerotium sp, Aspergillus sp and Rhizoctonia sp*) [3].

CONCLUSION

In our study, quinolizidine alkaloids of *R. monosperma* (leaves and stems) displayed very significant reduction in the growth of *C. albicans* and *C. tropicalis.* The most abundant compounds in alkaloids extracts of leaves and stems, namely sparteine, ammodendrine and anagyrine might be responsible for their antifungal activities. In the other hand, the high percentages of cytisine and its derivatives in alkaloids extract of seeds of *R. monosperma* might be responsible for its inactivity. To the best of our knowledge, for the first time, we herein report the antifungal activity of alkaloids extracts of *R. monosperma* that has traditional uses in Morocco.

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