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Phytochemical and antimicrobial screening of *Linaria reflexa* Desf.

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Abstract
In continuation of our investigations on the genus *Linaria* (Scrophulariaceae) and especially on the spice *Linaria reflexa* Desf., we have present in this paper, the phytochemical screening, the structural determination of (+)-pinoresinol (**1**) and antirrhide (**2**) together with the antimicrobial activity face to bacterial and fungal strains: *Escherichia coli*, *Bacillus sp*., *Yarrowia sp*. and *Candida sp*. of the aerial parts of *L. reflexa*. Good presences were observed for alkaloids, flavonoids, leucoanthocyanins and Tannins. No effects were observed about the antibacterial activity, while the PE, AcOEt and *n*-BuOH extracts exhibits antifungal activity against *Candida sp*. This is the first report describing (+)-pinoresinol in the Scrophulariaceae family and the antimicrobial activity of *L. reflexa*.

Keywords: phytochemical screening, antimicrobial activity, *Linaria reflexa*, Scrophulariaceae

Introduction
The genus *Linaria* belongs to the Scrophularioideae-Antirrhineae tribe of the Scrophulariaceae family and comprises about 200 species. Some *Linaria* species are used in folk medicine for treating various diseases in many geographical areas. Fresh or dried flowering of *L. vulgaris* is internally administered to treat digestion problems and urinary disorders. Externally, the plant is employed in the treatment of haemorrhoids, ulcus cruris, for ablution of festering wounds and skin rashes. It was also reported to have anti-inflammatory effect and to treat coughs and asthma. *L. japonica* is known under the name 'unran' used in the folk medicine as diuretic, purgative and laxative. *L. cymbalaria* is used for its diuretic, tonic and anti-scorbutic effects, and *L. reflexa* has been found to be applied in the North African folk medicine in the treatment of certain skin diseases [1]. Previous works on this genus has shown the presence of iridoids, diterpenoids, flavonoids, alkaloids and phenylethanoids [2-7]. Important cytotoxic and acetyl-cholinesterase inhibition effects were reported for neo-clerodane diterpenoids and flavonoids previously isolated respectively from *L. saxatilis* var. *saxatilis* and *L. reflexa* Desf. [8-10].
In continuation of our research on the genus *Linaria* [11-13], we present in this paper, the phytochemical screening, the isolation of two compounds and the antimicrobial activity of PE, $CHCl_3$, AcOEt, *n*-BuOH and MeOH extracts of the aerial parts of *Linaria reflexa* Desf.

2. Materials and methods
2.1. Plant material
The aerial parts of *Linaria reflexa* Desf., were collected in March 2012 from El-Meridj near Constantine city, Algeria. The plant was identified by Dr. D. Sarri on the basis of Quezel and Santa [14]. A voucher specimen

has been deposited in the Herbarium of the *VARENBIOMOL* Unit Research, Université des Frères Mentouri under n° 06/2012/SLR.

2.2. Preliminary phytochemical screening
The dried aerial parts of *L. reflexa* were subjected to preliminary phytochemical screening to identify the various active chemical constituents present in this spice such as alkaloids, coumarins, terpenoids, triterpenoids, unsaturated sterols, saturated sterols, anthocyanins, leucoanthocyanins, flavonoids, saponins and tannins according to standard phytochemical methods as described by Harborne [15] and khandelwal [16].

2.3. Extraction and isolation
Dry aerial parts of *L. reflexa* (1500 g) were macerated with 80% aqueous methanol at room temperature four times. After filtration, the filtrate was concentrated and dissolved in water (600 ml). The resulting solution was extracted successively with PE (1 × 200 ml), $CHCl_3$ (3 × 200 ml), EtOAc (3 × 200 ml) and *n*-BuOH (3 × 200 ml). Combined solutions were concentrated under reduced pressure and dried (PE: 3 g, 0.23%; $CHCl_3$: 5 g, 0.33%; EtOAc: 7 g, 0.54%; *n*-BuOH: 45 g, 3%). 15 g of the aerial parts were extracted by ultrasound method using methanol to obtain 3.5 g (23.33%). The chloroform extract has been fractioned by silica gel flash column chromatography eluted with *n*-hexane containing increasing percentages of EtOAc to obtain 10 fractions. The 4th fraction was subjected to PLC with *n*-hexane/EtOAc (7/3) to isolate compound **1** (5.2 mg). The MeOH extract was fractioned by RP-18 column chromatography starting with MeOH/H_2O (2/8) to 100% MeOH to obtain 25 fractions (F1-F25). F10 was subjected to silica gel column chromatography with $CHCl_3$/isopropanol to obtain 12 sub-fractions. F10-9 (67 mg) was separated with PLC ($CHCl_3$/MeOH 85/15) to obtain compound **2** (5 mg).

2.4. Structural characterization of isolated compounds
NMR spectra were recorded on a Bruker-Avance 400 spectrometer by using a 5-mm BBI probe ^1H at 400 MHz and ^{13}C at 100 MHz in $CDCl_3$ (δ_H = 7.26 and δ_C = 77.16 ppm) and CD_3OD (δ_H = 3.31 and δ_C = 49.00 ppm), δ values in ppm, J values in Hz. Structural assignments are from correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments.
Electrospray ionization (ESI) mass spectra were taken by using a Bruker Esquire-LC spectrometer with an electrospray ion source used in positive or negative ion mode by direct infusion of a methanolic solution of the sample, under the following conditions: source temperature 300 °C, drying gas N_2, scan range 100–1,000 *m/z*.

2.5. Antimicrobial activity
In order to determine the antimicrobial activity of *L. reflexa*, *In vitro* antibacterial and antifungal activities were examined for the five extracts.

2.5. a. Test microorganisms
Two bacterial and fungal strains were used in this study in which one bacteria was Gram positive (*Bacillus sp.*) and the other one was Gram negative (*Escherichia coli*). However the fungal strains were two yeasts (*Yarrowia sp*. and *Candida sp*.). All microorganisms were obtained from Laboratory of Mycology, Biotechnology and Microbial Activity (*LaMyBAM*), University Frères Mentouri, Algeria.

2.5. b. Culture media
Nutrient Agar media (GN: peptone 10 g, Beef extract 5 g, Sodium chloride 5 g, Agar 15 g; distilled water 1L), Mueller Hinton Agar (MH: Beef, infusion from, 300 g; Casein acid hydrolysate, 17.5 g; Starch, 1.5, Agar, 17 g; distilled water 1L) and Yeast Starch Agar (YSA: Yeast extract, 4g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; Soluble starch, 15 g; agar, 20 g; 1/4L water; 3/4L distillated water).
The antibacterial activity was evaluated according to the plate diffusion method [17]. Bacterial organisms were grown on GN and MH for 24 h in plats at 37°C for *E. coli* and 30°C for *Bacillus sp*. However, the antifungal activity was investigated using plate diffusion method on YSA for *Yarrowia sp*. and *Candida sp*. this method was based on diffusion capacity of test chemicals through agar medium [18]. The plates were then incubated for 48 h to 72 h. The diameter of zone of inhibition of all extracts and activities were measured.

3. Results and discussion

3.1. Preliminary phytochemical screening

Plants are important source for discovering new therapeutically natural products. the present study was aimed to focus on the various phytochemical constituents from of the aerial parts of *L. reflexa*.
Table 1 shows the phytochemical screening of the aerial parts of *L. reflexa*, this analyse has revealed the high presence of alkaloids, leucoanthocyanins and flavonoids. Good presence has been detected for anthocyanins and saponins and some traces of terpenoids and unsaturated sterols, while negative results were observed for coumarins, triterpenoids and saturated sterols. This knowledge could be used for identifying the various biological potentials of this plant.

Table 1: Chemical composition of the petroleum ether extract of *L. reflexa*

Chemical groups	Aerial parts	Chemical groups	Aerial parts
Alkaloids	+++	anthocyanins	++
Coumarins	-	leucoanthocyanins	+++
Terpenoids	±	flavonoids	+++
Unsaturated sterols	±	saponins	++
triterpenoids	-	Tannins cathechics	+++
Saturated sterols	-		

"+" Presence, "±" traces, "-" Absence.

3.2. Identification of isolated compounds

The ESI mass spectra of compound **1** gave a molecular ion signals in positive mode at *m/z* 381 $[M+Na]^+$, and in negative mode at *m/z* 357 $[M-H]^-$consistent with the formula $C_{20}H_{22}O_6$. The ESI(-)MS/MS spectrum indicated the presence of free hydroxyl and methoxyl groups, this information was confirmed by ^{1}H NMR spectrum. The ^{1}H NMR and ^{13}C NMR showed a clear symmetric structure determinates by the presence of three aromatic proton signals at δ_H = 6.88, 6.87 and 6.81 ppm interpreted to H-2 H-2', H-5 H-5' and H-6 H-6' respectively. The correlations between the last mentioned protons and C-3, C-3' (δ_c = 146.6 ppm) in HMBC spectrum together with protons of the methoxyl group (δ_H = 3.89 ppm, 2 × OMe) prove the substitution of these two on C-3 and C-3'. Carbons C-4, C-4' (δ_c = 145 ppm) are substituted by two OH groups. A correlation between H-2 H-2', H-5 H-5', H-6 H-6'and a quaternary carbon at δ_c = 132.8 ppm which correlated also with a doublet at δ_H = 4.73 ppm interpreted to H-7 H-7' and the quaternary carbon was determined as C-1 C-1'. The protons H-7 H-7' correlate with H-8 H-8' (δ_H = 3.08 ppm), H-9a H-9'a (δ_H = 4.24 ppm) and H-9b H-9'b (δ_H =3.87). The treatment of all MS and NMR (1 & 2D) data led the identification of compound **1** as (+)-pinoresinol, this compound was isolated for the first time from the Scrophulariaceae family [19].
(+)-pinoresinol (**1**): yellow amorphous; $C_{20}H_{22}O_6$; ESI(+)-MS *m/z* 381.2 $[M+Na]^+$, 403.1 $[M+COOH]^+$, ESI(-)-MS *m/z* = 357.4 $[M-H]^-$, ESI(-)MS/MS (357.1) *m/z* 342, 311, 175, 150.9, 136; ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.88 (2H, d, J = 2 Hz, H-2, 2'), 6.81 (2H, dd, J = 2, 8 Hz, H-6, 6'), 6.87 (2H, d, J = 8 Hz, H-5, 5'), 4.73 (2H, d, J = 4 Hz, H-7, 7'), 4.24 (2H, dd, J = 6.4, 8.8 Hz, H-9a, 9'a), 3.89 (6H, s, 3-OCH_3, 3'-OCH_3), 3.87 (2H, dd, J = 3.2, 8.8 Hz, H-9b, 9'b), 3.08 (2H, m, H-8, 8'); ^{13}C NMR (100 MHz, $CDCl_3$): 132.8 (C-1, 1'), 108.4 (C-2, 2'), 146.6 (C-3, 3'), 145 (C-4, 4'), 114.1 (C-5, 5'), 118.8 (C-6, 6'), 85.6 (C-7, 7'), 53.9 (C8, 8'), 71.4 (C-9, 9'), 55.8 (3-OCH_3, 3'-OCH_3).

The ^{1}H NMR spectrum of compound **2** showed the presence of signals corresponding to an iridoid structure by the presence of the doublet at δ_H = 6.16 ppm determined as H-3, this proton correlate in the COSY spectrum with the signal of H-4 (δ_H = 4.72 ppm) and this one correlate with a multiple at δ_H = 2.86ppm which is H-5. The last mentioned proton has shown 2 correlations, the first one with two multiple signals at δ_H = 1.92 and 1.74 ppm attributed to H-6α and H-6β respectively, and the second one with another multiple at δ_H = 2.96 ppm which is the H-9. This proton was confirmed by the correlation with H-1 (δ_H = 5.47 ppm) in COSY and C-1 (95.4), C-5 (30), C-8 (153.6) and C-10 (111.1) in HMBC. The proton H-5 correlate in COSY with two signals determinates to be H-6α (δ_H = 1.92 ppm) and H-6β (δ_H = 1.74 ppm) confirmed by the correlation of these two protons to the same carbon in the HSQC spectrum (C-6, δ_c = 40.9 ppm). H-6α and H-6β correlates with the H-7 at δ_H = 4.53 ppm. The correlation between the two protons H-10α and H-10β and C-8 confirmed

that this CH_2 is substituted on C-8. The NMR and mass data confirms the structure of compound **2** as antirrhide. This compound is very well known in the genus and the spice [8].
Antirrhide (**2**): white amorphous; $C_{15}H_{22}O_8$; ESI(+)-MS *m/z* 353 $[M+Na]^+$, 683 $[2M+Na]^+$, ESI(-)-MS *m/z* = 329.5 $[M-H]^-$, 365.5 $[M+Cl]^-$, ESI(-)MS/MS (353.1) *m/z* 335.1, 283; ^{1}H NMR (400 MHz, CD_3OD, δ, ppm, J/Hz): 6.16 (1H, d, J = 6.4, 1.8 Hz, H-3), 5.47 (1H, dd, J = 2.4 Hz, H-1), 5.33 (1H, d, J = 2 Hz, H-10*α*), 5.26 (1H, d, J = 2 Hz, H-10*β*), 4.72 (1H, dd, J = 6.3, 1.2 Hz, H-4), 4.66 (1H, d, J = 8 Hz, H-1'), 4.53 (1H, t, J = 2.4 Hz, H-7), 3.86 (1H, d, J = 12 Hz, H-6'*α*), 3.67 (1H, dd, J = 12, 4.8 Hz, H-6'*β*), 2.96 (1H, m, H-9), 2.86 (1H, m, H-5), 1.92 (1H, m, H-6*α*), 1.74 (1H, m, H-6*β*); ^{13}C NMR (100 MHz, CDCl3): 95.4 (C-1), , 140.9 (C-3), 107.5 (C-4), 30 (C-5), 40.9 (C-6), 74.9 (C-7), 153.6 (C8), 45.3 (C-9), 111.1 (C-10), 99.5 (C-1'), 74.3 (C-2'), 78.3 (C-3'), 71.7 (C-4'), 78 (C-5'), 62.8 (C-6').

Figure 1: (+)-pinoresinol (**1**) and antirrhide (**2**) structures

3.2. Antimicrobial activity
Antibacterial and antifungal potential of extracts were assessed in terms of inhibition zone of bacterial and fungal growth. The results of the antibacterial and antifungal activities are presented in table 2.
The evaluation of the antimicrobial activity of five extracts against two bacterial strains (Gram positive: *Bacillus sp*., and Gram negative: *Escherichia coli*) and two fungal strains (*Yarrowia sp.* and *Candida sp.*) showed that $CHCl_3$ and *n*-BuOH extract has no activity against bacteria's or fungus and this may be related to the chemical composition of these extracts with the presence of terpenoids determinated previously by the phytochemical screening. However, in the case of PE extract, we observed good results against fungus, Gram – bacteria and nothing against Gram + bacteria strain. Both AcOEt and MeOH extracts had no activity against the tested bacteria strains but they showed good results when faced with the fungal strains especially *Candida sp*. Some papers suggest that the presence of good amount of flavonoids and alkaloids is good for the antifungal activity especially against *Candida sp.,* which is our case [20-26].

Table 2: Antimicrobial activity of five extracts of *L. reflexa*

extracts	Diameter of zone of inhibition (mm)			
	Microorganisms			
	E. coli	*Bacillus sp.*	*Yarrowia sp.*	*Candida sp.*
PE	+- (1 mm)	- (0 mm)	+ (6 mm)	+ (6 mm)
$CHCl_3$	+- (1 mm)	- (0 mm)	- (0 mm)	- (0 mm)
AcOEt	- (0 mm)	+- (1 mm)	+ (4 mm)	+ (4 mm)
n-BuOH	- (0 mm)	- (0 mm)	- (0 mm)	- (0 mm)
MeOH	- (0 mm)	- (0 mm)	+- (1 mm)	+ (6 mm)

Conclusion
This study on the aerial parts of *L. reflexa* which is a folk medicine herb in North Africa is about the phytochemical and antimicrobial screening looking for chemical composition and its relationship with the biological effect, this phytochemical analysis showed that this spice is loaded in flavonoids, alkaloids and tannins, and this confirmed the observed antifungal activity. In the phytochemical investigation, we described the isolation of (+)-pinoresinol which is new for the Scrophulariaceae family.

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