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Chemical constituents and antimicrobial activities of Petroleum ether and *n*-butanol extracts from *Linaria tingitana* Boiss. & Reut.

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Abstract
In continuation of our phytochemical studies on the genus *Linaria* (Scrophulariaceae), we have investigated the petroleum ether (PE) and *n*-butanol (*n*-BuOH) extracts from the aerial parts of *Linaria tingitana* Boiss. & Reut. In this paper we describe the determination of nine methyl esters in the petroleum ether by GC-MS and the LC-MS profile of the *n*-BuOH extract together with the antimicrobial activity (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus sp*., *Alternaria sp*. and *Yarrowia sp*.), both extracts exhibits antibacterial activity against *Staphylococcus aureus* and *Bacillus sp*., while no effects were observed about the antifungal activity. These results are reported for the first time for the spice.

Keywords: GC-MS, LC-MS, methyl esters, antimicrobial activity, *Linaria tingitana*, Scrophulariaceae

Introduction
The genus *Linaria*, one of 220 genus's of the scrophulariaceae family, comprises about 200 species [1], widely distributed in the Mediterranean basin and Eastern Asia. In Algeria, this genus has a good presence with 39 species and sub-spices [2]. The use of some *Linaria* species in folk medicine in many area for treatment of various maladies, for example fresh or dried flowering of *L. vulgaris* is used internally to aid digestion problems and urinary disorders. Externally, the plant is applied in the treatment of hemorrhoids, ulcuscruris, for ablution of festering wounds, and skin rashes. It is also reported to have anti-inflammatory effect [3] and to treat coughs and asthma [4]. *L. japonica* known under the name "unran" used in the folk medicine as diuretic, purgative [5] and laxative [6]. *L. cymbalaria* is used for their diuretic, tonic and antiscorbutic effects [7]. *L. reflexa*Desf., an North African folk medicine herb used for the treatment of certain skin diseases [8]. Other species have been used as tonics, antiscorbutics, laxatives, antidiabetics and diuretics, as well as for the treatment of wounds, hemorrhoids and vascular disorders [7; 9]. Previous researches on the genus showed the presence of many secondary metabolites mainly iridoids, flavonoids, diterpenoids, acyclic monoterpenoids, triterpenoids and alkaloids [4; 10-19]. Many biological effects have been reported for some isolated compounds like the neo-clerodane diterpenoids and flavonoids previously isolated respectively from *L. saxatilis* var. *saxatilis*and *L. reflexa*Desf., showed cytotoxic activity [13; 20]. Flavones and extracts of *L. reflexa*Desf., showed acetyl-cholinesterase inhibition [21]. Extracts of *Linaria* spices showed antibacterial [22], anti-inflammatory and analgesic [23], anti-oxidant [24] and α-glucosidase inhibitory [25] effects.

In continuation of our research on the genus *Linaria* [26], we present in this paper, the phytochemical and the antimicrobial screening of PE and *n*-BuOH extracts of the aerial parts of *Linaria tingitana* Boiss. &Reut., an endemic spice for Algeria.

2. Materials and methods

2.1. Plant material

The aerial parts of *L. tingitana* Boiss. &Reut., were collected during the flowering phase in May 2010 from the national park of El-Kala, Algeria. The plant was authenticated by Dr. Sarri Djamel on the basis of Quezel and Santa [2]. A voucher specimen (No. 08/2009/CCN12) has been deposited in the Herbarium of the *VARENBIOMOL* unit research, University of Constantine1. No previous phytochemical or pharmacological properties have been reported.

2.2. Extracts preparation

Dry aerial parts of *L. tingitana* (1285 g) were macerated with 80% aqueous methanol at room temperature four times. After filtration, the filtrate was concentrated and dissolved in water (514 ml). The resulting solution was extracted successively with PE (1 × 200 ml), $CHCl_3$ (3 × 200 ml), AcOEt (3 × 200 ml) and *n*-BuOH (11 × 200 ml). Combined solutions were concentrated under reduced pressure and dried (PE: 3 g, 0.23%; $CHCl_3$: 8 g, 0.62%; AcOEt: 7 g, 0.54%; *n*-BuOH: 61g, 4.74%).

2.3. GC-MS analysis

The PE extract was investigated by capillary gas chromatography–mass spectrometry (GC-MS). An Agilent gas chromatograph Model 7890, coupled to a Agilent MS model 5975, equipped whith a DB5 MS column (30 m 0.25 mm, 0.25mm), programming from 50°C (5 min) to 300°C at 5°C 1 min - 5 min hold. Helium was used as carrier gas (1.0 ml min-1); injection was in split mode (1:100); injector and detector temperatures were 250°C and 280°C, respectively.

2.4. LC-MS analysis

The *n*-BuOH extract of *L. tingitana* was subjected to the LC-MS screening looking for known secondary metabolites of the genus *Linaria*. The profile (Figure 2) was performed on a Hewlett–Packard (Palo Alto, CA, USA) Model 1100 Series liquid chromatograph coupled to a Photo Diode Array detector (Agilent, Palo Alto, CA, USA) 1100 Series, and to an Esquire LC–ion trap mass spectrometer (BrukerDaltonics, Billerica, MA, USA) equipped with an electrospray ionisation (ESI) interface. The Photo Diode Array detector was set at 200-700 nm and the UV-chanel at 204, 234, 254, 470, 665 nm. The system was A=H_2O + 0.1% formic acid, B= CH_3OH + 0.1% formic acid, with gradient: A=80% to 0% 40 min of analysis, the split of the column effluent was used to achieve a flow rate of 1 ml/min into the mass spectrometer. The LC-MS was run on a phenomenexKinetex C18 (250 mm x 4.6 mm i.d.; 5 µm particle diameter, end-capped). High-purity nitrogen was used as the nebulizer, also as the drying gas at 300 °C at a constant flow rate of 6 l/min. Full scan spectra were acquired in negative and positive ion mode in the region m/z 100–1000, adopting the following parameters: trap drive units, 55.1; capillary exit voltage, –120.4 V; skimmer 1 voltage, –43.3 V. MS/MS fragmentation experiments were performed on the selected precursor ion. Data Analysis (Version 3.0, BrukerDaltonik GmbH) was used to analyze the mass spectra.

2.5. Antimicrobial activity

In order to determine the antimicrobial activity of *Linaria tingitana*, *In vitro* antibacterial and antifungal activities were examined for petroleum ether and *n*-BuOH extracts.

2.5.a. Test microorganisms

Three bacterial strains and two fungal strains were used in this study in which two bacteria were Gram positive (*Staphylococcus aureus* and *Bacillus sp*.) and one Gram negative (*Escherichia coli*). However the fungal strains were one yeast (*Yarrowia sp*.) and one mold (*Alternaria sp*.). The microorganisms *Escherichia coli* and *Staphylococcus aureus* where obtained from the institute of Pasteur, Algeria. While *Bacillus sp*., *Yarrowia sp*. and *Alternaria sp*. where obtained from Laboratory of Mycology, Biotechnology and Microbial Activity (*LaMyBAM*), University Constantine 1, 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), 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), Potato Dextrose Agar (PDA: D-glucose, 20 g; Potato extract, 200 g; Agar, 20 g; distilled water 1L).

The antibacterial activity was evaluated according to the plate diffusion method [27]. Bacterial organisms were grown on GN and MH for 24 h in plats at 37°C for *E. coli*, *Staphylococcus aureus* and 30°C for *Bacillus sp*. However, the antifungal activity was investigated using plate diffusion method on YSA for *Yarrowia sp*. and on PDA for *Alternaria sp*. this method was based on diffusion capacity of test chemicals through agar medium [28]. The plates were then incubated at 30°C between 48 h to 72 h. The diameter of zone of inhibition of both extracts and activities were measured.

3. Results and discussion

3.1. Preliminary phytochemical screening

Using different chromatographic methods, we have investigates two PE and *n*-BuOH extracts from the aerial parts of *L. tingitana* looking for their chemical composition. The PE extract was investigated with GC-MS method, fifteen peaks were detected while nine were determinates as methyl esters (Figure 1; Table 1). Methyl palmitate (MP, methylester of palmitic acid, 53.83 %), Methyl oleate (MO, methylester of oleic acid, 21.28 %) and Methyl stearate (MS, methylester of stearic acid, 15.81 %) are the major constituents of PE extract. Methyl palmitate is an endogenous naturally occurring fatty acid methyl ester [29]. This major compound (MP) known for many biological activities like androgenic activity [30], anti-inflammatory [31-33], antioxidant and anti-fibrotic effects [33]. No previous research recorded the antimicrobial of MP or MO but many oils and plants extracts containing these two compounds knowing for good antimicrobial properties [34-36].

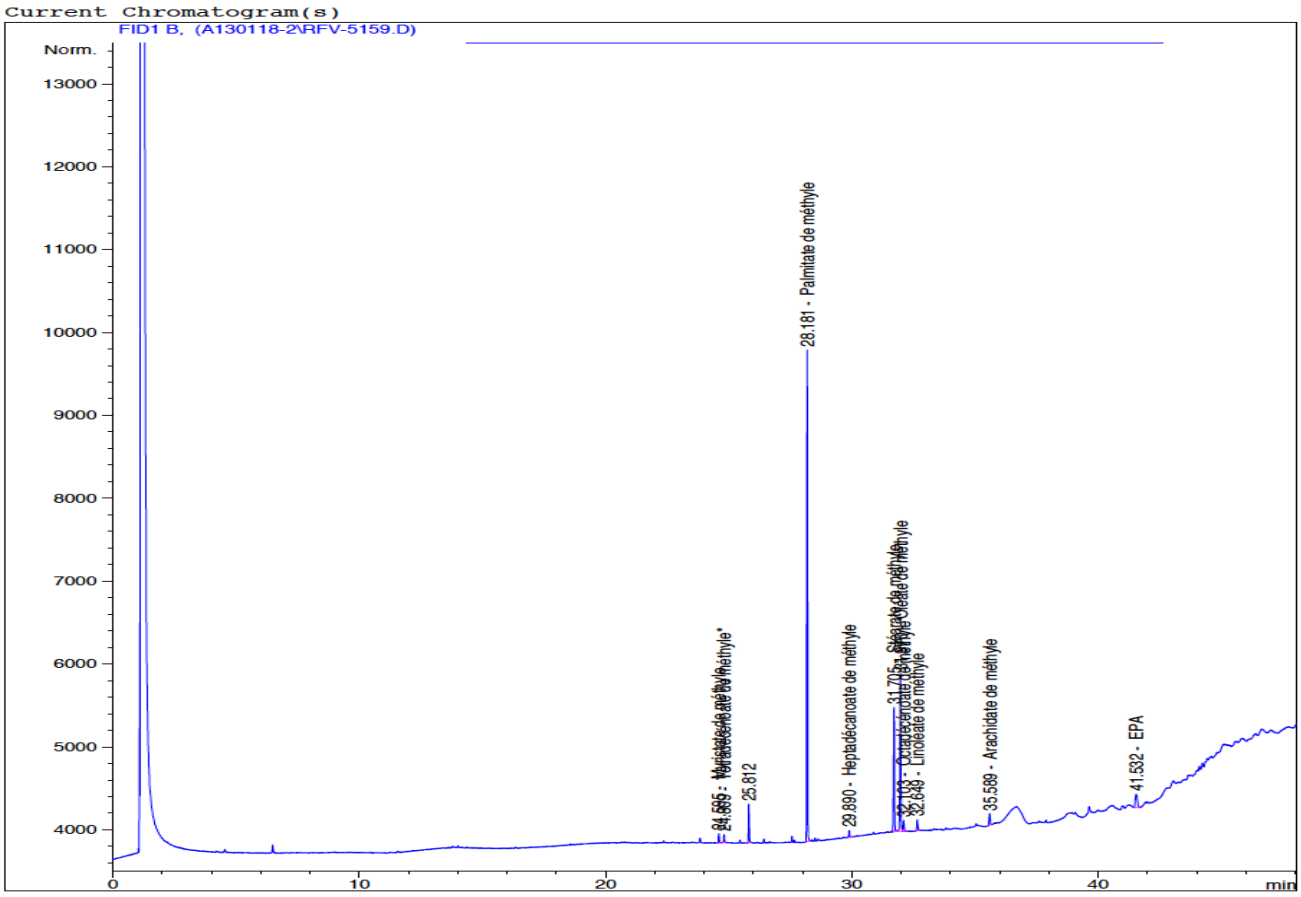

Figure 1: GC-MS profile of PE extract.

Using another chromatographic method, we have investigate the *n*-BuOH extract with LC-MS using a known profile for flavonoids, many constituents (figure 2) were detected but we are unable to determinate their structures. We think that the present constituents most of them are flavonoids and terpenoids.

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

Compound	R_t(min)	%
Methyl myristate	24.595	1.04812
Methyl tetradecenoate	24.809	0.99960
Methyl palmitate	28.181	**53.83782**
Methyl heptadecanoate	29.890	0.83461
Methyl stearate	31.705	**15.81679**
Methyl oleate	31.970	**21.28226**
Methyl octadecenoate	32.103	1.48831
Methyl linoleate	32.649	1.69678
Methyl arachidate	35.589	1.78081
Total		98.7851

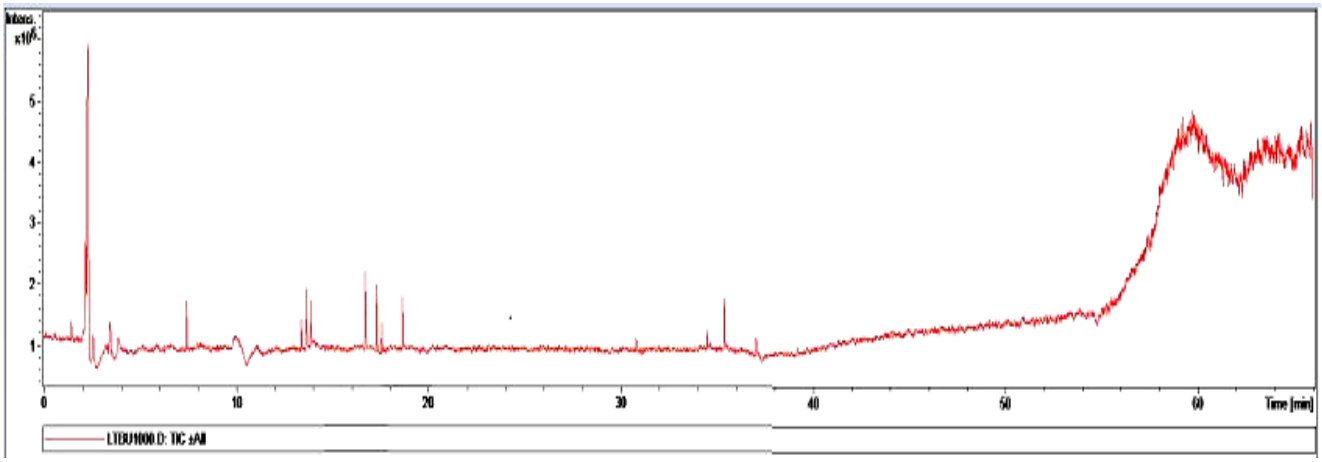

Figure 2: LC-MS profile of the *n*-BuOH extract

3.2. Microbial activity

Antibacterial and antifungal potential of extracts were assessed in terms of inhibition zone of bacterial growth. The results of the antibacterial and antifungal activities are presented in table 2.

The evaluation of the antimicrobial activity of both extracts PE and *n*-BuOH against bacterial strains with two Gram positive (*Staphylococcus aureus* and *Bacillus sp*.), one Gram negative (*Escherichia coli*) and two fungus (*Yarrowia sp*. and *Alternaria sp*.) showed that both extracts develop fairly well activity against Gram positive strains, especially against *Bacillus sp*., (figure 3) but no activity was observed against Gram negative strain or against fungus. Calce et al. [37] showed that among the tested fatty acids, oleate and linoleate acids exhibited the better antimicrobial activity against Gram positive bacteria (*S. aureus*) compared with Gram negative bacteria. In another study, various fatty acids of *Agaricus* species did not show any antibacterial activity against Gram-negative bacteria at test concentration; however, Gram-positive bacteria were inhibited by these extracts [38].

Various studies have investigated the antimicrobial effect of different plants against bacterial and fungal strains [39-43]. Nevertheless, to our knowledge, no study has been reported concerning the antimicrobial activity of *Linaria tingitana*. Only one previous research presents the antimicrobial activity of *L. corifolia* Desf. [22], the author mentioned that the ethanol extract of this spice showed a good antibacterial activity against Gram positive but no significant activity was found against Gram negative which is very similar to our results. The chemical composition plays a role of the biological activity like in our case the genus *Linaria* is very well known for the presence of iridoids, diterpenoids and flavonoids but not common for alkaloids which make us understand the microbial activity results.

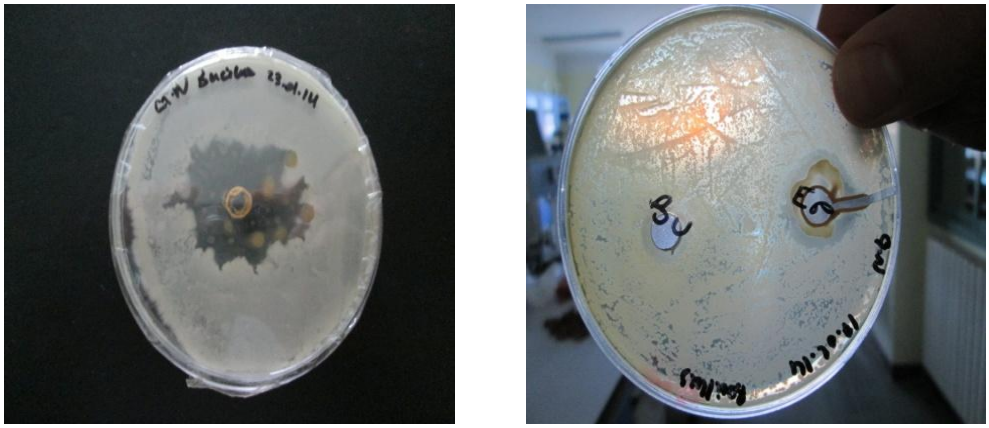

Figure 3: inhibition zone of PE and *n*-BuOH against *Bacillus sp*.

Table 2: Antimicrobial activity of PE and *n*-BuOH extracts of *L. tingitana*

extracts	Diameter of zone of inhibition (mm)				
	Microorganisms				
	E. coli (Gram -)	*S. aureus* (Gram +)	*Bacillus sp.*(Gram +)	*Alternaria sp.*	*Yarrowia sp.*
PE	0	9	10	0	0
n-BuOH	0	1	6	0	0

Conclusion

This study about one *Linaria* species, *L. tingitana* which is an endemic spice for Algeria present the chemical composition and antimicrobial activity of PE and *n*-BuOH extracts. Good results were observed against Gram positive bacteria which are in compatibility with a previous research on one *Linaria* species. This confirms the effect of the present compounds in the PE extract with previous researches on the methyl esters compositions.

The uses of the many *Linaria* species in the folk medicine need to be understood, and also to investigate the corresponding constituents for their biological effects, in Algeria which the genus *Linaria* is very distributed with 39 species and sub-species. The need for more researches about the biological effects and the chemical composition of *Linaria* genus is important, further study is needed on the selected plant.

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