Research Article

Phytochemical Screening, *In Vitro* Antimicrobial and Antioxidant Properties of *Linaria tingitana* Boiss. & Reut.

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ABSTRACT

To evaluate the potential of biological effects from the aerial parts of *Linaria tingitana* Boiss. & Reut., the chloroform ($CHCl_3$) and ethyl acetate (EtOAc) extracts were investigated using NMR 1 & 2D analysis, together with the phenolic and flavonoid levels. Antioxidant activity was determined by measuring the DPPH scavenging effect and metal ferrous-chelating ability. The antimicrobial activity was evaluated *in vitro* against to Escherichia coli, Bacillus sp., Yarrowia sp. and Candida sp. The phytochemical investigation of the EtOAc extract led the isolation of jaceosidin, this compound was isolate for the first time from this genus. The results showed that EtOAc extract had high level of polyphenols (61,61±0,89) (GAE)/g extract and flavonoids (59,61±0,89) (QE)/g extract, displayed highest antioxidant activity (IC_{50} :82,40±16 µg/mL) in the DPPH assay close to that of ascorbic acid and showing a high dose dependent manner ferrous chelating capacity compared to $CHCl_3$ which was the weaker extract. The antibacterial activity of the tested extracts was efficiency against some bacteria strains.

Keywords: Polyphenols, flavonoids, *Linaria tingitana*, antioxidant, antimicrobial.

INTRODUCTION

The use of plants as source of remedies for the treatment of many diseases dated back to prehistory, and many populations have this old tradition, because it represents a valuable source of new compounds. In recent years, significant efforts have been directed on plants and their by-products to extract natural and low-cost antioxidants,[1] which have an excellent source of chemical structures with a wide variety of biological activities[2] and can be beneficial in suppressing enhanced ROS production and thus may be useful for the prevention and mitigation of oxidative stress-related diseases. Among the dietary antioxidants, phenolic compounds, secondary metabolites occurring in plants, are the most abundant natural antioxidants.[3]

In 1987, World Health Organization (WHO) has stated the importance of scientific research on herbal supplements.[4] Thus, some researchers suggest that two-thirds of the world's plant species have medicinal value; in particular, many medicinal plants have great antioxidant potential.[5] Therefore, qualitative and quantitative determination of these compounds in plants extracts and evaluation of their biological activities are undoubtedly very important.

A review by Cheriet[6] has demonstrated the use of some *Linaria* species (Scrophulariaceae) in the folk medicine, the presence of many secondary metabolites classes especially iridoids, diterpenoids, flavonoids, phenylethanoids and alkaloids following by the pharmacological effects observation such as antioxidant[7] and antibacterial properties.[8]

In continuation of our research of this spice,9 the current study quantified the phenolic and flavonoid contents and both chloroform ($CHCl_3$-LT) and ethyl acetate (EtOAc-LT) extracts of the aerial parts from Linaria tingitana Boiss. & Reut. The antioxidant activity of $CHCl_3$-LT and EtOAc-LT was investigated based on their ability to scavenge non biological stable free radical (DPPH°), and to chelate metal ions. The antimicrobial activity was tested against two bacterial and fungal strains: *Escherichia coli*, *Bacillus sp*., *Yarrowia sp*. and *Candida sp*., to establish whether or not they have antimicrobial activity.

MATERIALS AND METHODS

Chemicals and reagent

1,1-diphenyl-2-picrylhydrazyl (DPPH), Gallic acid, Folin-Ciocalteus's phenol reagent, quercetin, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), Ethylenediaminetetraacetic acid (EDTA), Aluminium chloride ($AlCl_3$), Ferrous chloride ($FeCl_2$), Ferrozine and sodium carbonate (Na_2CO_3). The solvents and/or reagents were of Analytical Grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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.[10] A voucher specimen (No. 08/2009/CCN12) has been deposited in the Herbarium of the

VARENBIOMOL unit research, Université des Frères Mentouri Constantine.

Preparation of plant extract

The dried aerial parts of *L. tingitana* (1285 g) were macerated using a hydroalcoholic solution containing MeOH-H_2O (80-20) at room temperature four times during 72 h. After filtration, the filtrate was concentrated and dissolved in water (514 ml). The resulting solution was extracted successively with petroleum ether (1×200 ml), $CHCl_3$ (3×200 ml), EtOAc (3×200 ml) and *n*-BuOH (11×200 ml). Combined solutions were concentrated under reduced pressure and dried (PE: 3 g, $CHCl_3$: 8 g, EtOAc: 7 g, *n*-BuOH: 61 g). In our investigation we are interested to both $CHCl_3$ and EtOAc extracts.

Structural characterization of jaceosidin

NMR spectra were recorded on a Bruker-Avance DP 250 spectrometer by using a probe ^1H at 250 MHz and ^{13}C at 62.9 MHz in CD_3OD (δ_H = 3.31 and δ_C = 49.00 ppm), δ values in ppm, J values in Hz. UV data was recorded on Thermo Nicolet evolution l 300 spectrometer using methanol as solvent.

Antioxidant activity determination

Each sample was dissolved in 95% methanol to make a concentration of 1 mg/ml and then diluted to prepare the series concentrations for assays and reference chemicals (Ascorbic acid, Trolox, Quercetin) were used for comparison.

DPPH radical scavenging activity

The effect of $CHCl_3$-LT and EtOAc-LT on DPPH radical was assayed using the method of Ohinishi.[11] with minor modifications. 1 ml of a methanolic solution of DPPH (0.2mM) was added to 1 ml of the different concentrations of both extracts and allowed to react in the dark at room temperature for 30 min. Methanol served as the blank and DPPH in methanol without the extract served as the control sample. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid, Trolox and quercetin were used as references. Results were expressed as percentage of inhibition of the DPPH radical according to the following equation:

$$\%\ Inhibition\ of\ DPPH = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Ferrous-chelating ability

The method of Dinis (1994)[12] was adopted for the assay except that ferrous sulphate was substituted for ferrous chloride. Briefly, 50 µl of $FeCl_2$ (2 mM) was added to various concentrations of both extracts (1 ml).

The reaction was initiated by the addition of 0.2 ml of ferrozine solution (5 mM). The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance was measured spectrophotometrically at 562 nm. EDTA was used as reference standard. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and Ferrozine only) using the formula:

$$Ferrous\ ion\ chelating\ ability(\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Total phenolic content (TPC) determination

We determined the total phenolic in $CHCl_3$-LT and EtOAc-LT using Folin-Ciocalteu reagent,[13] with slight modifications. 1000 µl of extract was mixed with 250 µl Folin-Ciocalteu reagent (1N) and allowed to stand at room temperature for 2 min. 1250 µl of sodium carbonate (20%) was added, then the mixture was mixed and allowed to stand at room temperature in the dark for 2 h. The absorbance was measured at 765 nm with a spectrophotometer. Quantitation was based on the standard curve of gallic acid (0-0.2 mg/ml). The results were expressed as gallic acid equivalents (GAE)/g extract.

Total flavonoid content (TFC) determination

Aluminum chloride calorimetric method was used for flavonoids determination.[14] A volume of 0.5 ml of 2% AlCl3 ethanol solution was added to 0.5 ml of sample solution.

After incubation at room temperature for 1 h, the absorbance was measured at 420 nm along with standard quercetin and blank. A yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.25 mg/ml. Total flavonoids content were calculated as quercetin (mg/g) using the calibration curve. Results were expressed as mg quercetin equivalents (QE)/g extract.

Statistical analysis

The experiments were performed in triplicate and results expressed as means ± standard deviation (SD). IC_{50}-value (µg extract/ml) is the effective concentration which proves 50% of activity, was calculated for each assay with respect to control against treatment concentration. Statistical comparisons were done with Student's test.

Antibacterial and antifungal activities

In order to determine the *in vitro* antimicrobial activity of *L. tingitana*, antibacterial and antifungal activities were examined for both extracts.

Microorganisms used

The antimicrobial activity of *L. tingitana* extract was evaluated on two common pathogenic bacteria *Bacillus sp.* (Gram positive) and *Escherichia coli* (Gram negative). The antifungal activity was evaluated on two common fungi *Yarrowia sp.* and *Candida sp.* (yeasts). This evaluation was done by well diffusion method.

All microorganisms were obtained from Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM), Université des Frères Mentouri, Algeria.

Culture media

Nutrient Agar media (GN: peptone 10 g, Beef extract 5 g, Sodium chloride 5 g, Agar 15 g; distilled water 1 l), Mueller Hinton Agar (MH: Beef, infusion from, 300 g; Casein acid hydrolysate, 17.5 g; Starch, 1.5, Agar, 17 g; distilled water 1 l) 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; ¼ l water; ¾ l distillated water).

Plate diffusion assay

To investigate the antibacterial activity, sterile Muller Hinton agar media was prepared in Petri dishes. The bacteria was inoculated separately in the media. In each Petri dish, four wells (diameter 6 mm) were prepared under sterilized conditions. Eighty microliter of each extract solutions prepared in DMSO was added to the wells and in one well, normal distilled water without extract was performed. The dishes were incubated at 37°C for 24 h then the zone of inhibition was measured. For the investigation of antifungal activity, Yeast Starch Agar were prepared in petri dishes. The fungal spores were inoculated separately in the media. In each petri dish, four wells (diameter, 6 mm) were prepared under aseptic conditions. Eighty microliter of each extract solutions prepared in DMSO was added to the wells and in one well, normal distilled water was added. The plates were incubated at 37°C for 24 h. DMSO is used as a blank. All the dishes were incubated at 30°C for 72 h. At the end of the incubation period, the zones of inhibition were measured.[15]

RESULTS AND DISCUSSION

Determination of antioxidant activity

Historically, medicinal plants were recognized by religious and cultural traditions for their use as health cures and functional foods, which can be related to the presence of bioactive phytochemicals, peptides and secondary metabolites.[16] These substances that play as antioxidants delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions.[17] The genus *Linaria* is known for the presence of some secondary metabolites classes especially iridoids, diterpenoids, flavonoids, alkaloids and phenylethanoids.[6]

In this study, the antioxidant and antimicrobial activities of $CHCl_3$-LT and EtOAc-LT were investigated. In which the antioxidant activity based on their ability to scavenge non biological stable free radical (DPPH), and chelate metal ions.

DPPH radical scavenging activity

In the presence of an antioxidant, DPPH radical form a stable purple colored free radical by gaining one more electron or hydrogen atom from the antioxidant and convert it into colorless α-α-diphenyl-β-picryl hydrazine. The UV absorbance decreases indicates the amount of DPPH scavenged which signifies the scavenging activity of natural products.[13] As shown in the Figure 1, both EtOAc-LT and $CHCl_3$-LT extracts have a remarkable potent radical scavenging activity with IC_{50}-values (82,40±16 µg/ml and 255,40±8,55 µg/ml) respectively (Table 1) which is close to that of ascorbic acid and trolox (11,44±0,32 % and 4,60±0,08 %) respectively. This ability to scavenge DPPH radicals suggests that they might be electron donors and react with free radicals to convert them to more stable products and terminate radical chain reactions which revealed the presence of antioxidant compounds in this plant.

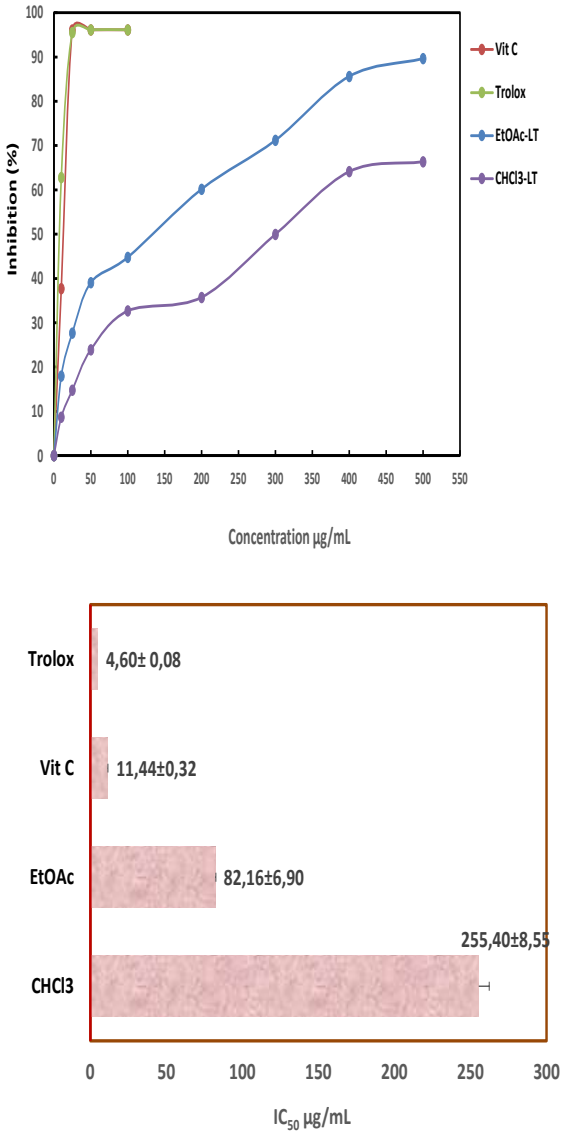

Figure 1: DPPH radical-scavenging activity of EtOAc-LT and $CHCl_3$-LT extracts and standards. Values are means ± SD (n=3) P<0.05.

Ferrous-chelating ability

The iron-chelating is used to measure the reductive ability of antioxidant, and it is evaluated ferrozine in chelating ferrous ion and the results are expressed as percentage metal chelating activity.[12] The investigated extracts compared to standard EDTA, is shown in Figure 2. The

Identification of isolated compound from EtOAc-LT

The ^{1}H NMR spectrum shown signals corresponding for flavonoid skeleton basing of the presence of aromatic signals at 7.52, 6.95, 6.58 and 6.46 ppm attributed to H-6', H-2', H-5', H-3 and H-8 respectively. The presence of two singlets with 3H as integration for each signal corresponding to two OMe groups located by UV spectra on C-4' and C-6. This data led the determination of this compound as 5, 7, 4'-trihydroxy-6, 3'-dimethoxyflavone (figure 1) knowing under the name jaceosidin[20] which is new for the genus *Linaria*.

Jaceosidin: yellow amorphous, mp 227-230 °C; $C_{17}H_{14}O_7$; UV (MeOH, λ_{max}, nm): 345, 275; +NaOH: 407, 334, 268; +$AlCl_3$: 368, 284, 258; +$AlCl_3$/HCl: 365, 284, 258; +NaOAc: 352, 276; +NaOAc/H_3BO_3: 348, 276; ^{1}H NMR (250 MHz, MeOD-d_4, δ, ppm, J/Hz): 7.52 (1H, dd, J = 8.1, 2.1 Hz, H-6'), 7.52 (1H, d, J = 2.1, H-2'), 6.95 (1H, d, J = 8.1, H-5'), 6.58 (1H, s, H-3), 6.46 (1H, s, H-8), 3.98 (3H, s, OCH_3), 3.87 (3H, s, OCH_3).

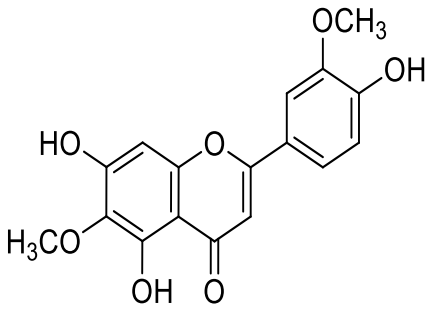

Figure 3: Jaceosidin

Antimicrobial activity

Antibacterial and antifungal potential of EtOAc-LT and $CHCl_3$-LT extracts were assessed in terms of inhibition zone of bacterial growth.

The results illustrated in Table 2 demonstrated that EtOAc-LT exhibit effect on *E. coli* (Gram positive) and no effect on *Bacillus sp.* (Gram positive). While $CHCl_3$-LT has no effect on *E. coli* (Gram negative) and effect on *Bacillus sp*., the two extracts show no effect on the tested fungi.

The structural differences of the bacterial strains and the discrepancy between the extract's constituents may play an important role in the bacterial response.

The antibacterial effect of EtOAc-LT could be attributed to the presence of methoxylated Jaceosidin isolated for the first time from EtOAc-LT.

CONCLUSION

In the present study, both extracts of *L. tingitana* are rich in Polyphenols and flavonoids showed a potent antioxidant activity in DPPH and Ferrous-chelating ability tests and exhibited an antibacterial effect towards some strains. These results let us to suggest that this plant would be a promising source of natural antioxidants and could offered scientific proof to the medicinal and commercial needs.

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