Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Antibacterial and antioxidant activities of endophytic fungi and nettle (*Urtica dioica* L.) leaves as their host

Djouza Salmi[1], Catherine Riou[3], Mohammad Issawi[3], Yacine Titouche[1], Veronica Ambrosini[3], Noria Smail-Saadoun[2], Hocine Abbaci[4], Karim Houali[1*]

[1] LABAB Laboratory, Faculty of Biological and Agronomic Sciences, Mouloud Mammeri University of Tizi-Ouzou, Algeria
[2] Natural Resources Laboratory, Faculty of Biological and Agronomic Sciences, Mouloud Mammeri University of Tizi-Ouzou, Algeria
[3] PEIRENE EA 75000, Faculty of Sciences and Technology, University of Limoges, France
[4] Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algérie

[*]**Correspondence to:** houalitizi@yahoo.fr

Received September 10, 2021; **Accepted** October 25, 2021; **Published** November 22, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.3.33

Abstract: Nettle (*Urtica dioica* L), as a plant rich in biologically active compounds, is one of the most important plants used in herbal medicine. Studies have shown that this plant has antioxidant, antiplatelet, hypoglycemic and hypocholesterolemia effects. In this study, we characterized three *Alternaria* endophytic fungi isolated from their host *U. dioica*. We hypothesized that these endophytic fungi can produce new bioactive metabolites, which may possess the bioactive property with potential application in the medical and pharmaceutical industries. The antibacterial activity was evaluated against reference and isolated strains, including Methicillin-Resistant *Staphylococcus aureus*. A wide range of antimicrobial activities similar to those measured in nettle leaves was detected especially for *Alternaria sorghi*. Furthermore, the highest antioxidant activity detected with DPPH free radical scavenging was measured for *A. sorghi* and nettle leaves ethyl acetate extracts. In addition, whereas catalase activity was similar in the three isolated fungi and nettle leaves, total thiol content and superoxide dismutase activity were significantly higher in leaves. *A. sorghi* showed the best activities compared to other isolated fungi. The characterization and further production of bioactive compounds produced by this endophyte should be investigated to fight bacteria and especially those that develop drug multi-resistance.

Key words: Antibacterial activity; Endophytic *Alternaria* species; Oxidative stress; Superoxide dismutase; Thiols.

Introduction

The emergence of antibiotic multi-resistant bacteria and new diseases for which modern medicine remains powerless led to a renewed interest intraditional medicine based on a solid and ancient knowledgeofmedicinal plants and their potential hosts. Indeed, medicinal plants produce thousands of molecules from secondary metabolism with therapeutic potential. Moreover, these plants often contain in their tissues or organs so-called endophytic organisms that offer their host resistance to biotic and abiotic stresses (1, 2). Furthermore, these endophytic fungi are known to promote the growth of the host plant and help it to defend itself against pathogens (2, 3). Thus, these fungi are able to produce various molecules with medical and pharmaceutical great interests such as antioxidant, anticancer, antiviral, immunomodulatory or molecules against tuberculosis or plant or animal parasites (1, 2, 4). Moreover, endophytic fungi were also shown to increase the activity of ROS detoxification enzymes such as catalase (CAT), superoxide dismutase (SOD) and peroxidases (POD), which promote the host plant's resistance to various stresses such as drought and soil salinity (5, 6).

Our study was focused on the world's widespread and medicinal Nettle (*Urtica dioica* L.) that also shelters endophytic fungi. To our knowledge, this is the first study of its kind to have studied both the biological activities of *Urtica dioica* leaves and endophytic colonizing fungi. This study aims to evaluate the antibacterial and antioxidant activity, the content of phenolic compounds and total thiols as well as the activity of ROS detoxifying enzymes (CAT and SOD).

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Germany), Biochem Chemopharma (Quebec), Bio-Red (France), Liofilchem (Italy) or Biokar (France).

Plant material and leaf extract preparation

Nettle (*Urtica dioica L.*) was identified and collected from Larba Nath Irathen (Tizi-Ouzou, Algeria). Fresh leaves were immediately washed with water. The leaves were shade-dried at 40°C for two to three days and powdered. Then, 20g powder wasmixed with 200 mL of water or ethyl acetate for 24h. The extracts were filtered on Whatman No.1 paper. The aqueous filtrate was frozen and lyophilized at a pressure of 5 mmHg at -50°C. The ethyl acetate filtrate was concentrated using a rotary vacuum evaporator at reduced pressure at 40°C. The nettle leaves extracts were then stored at -20°C until used.

Isolation of endophytic fungi

Healthy leaves were carefully selected for the isola-

tion of endophytic fungi. Leaves samples were washed thoroughly to remove adhered dust and debris. Under aseptic conditions, the leaf surfaces were sterilized by macerating in 96 % ethanol for 1 min, followed by soaking in 10 % sodium hypochlorite for 4 min and subsequently rinsed with ethanol for 30s. They were then washed in sterile distilled water and blot dried on sterile filter paper (7).

20 plants and 10 leaves per plant that were cut into four segments were placed aseptically on Petri dishes containing potato dextrose agar medium (PDA), supplemented with chloramphenicol (100 µg.mL^{-1}) to prevent bacterial growth. The Petri dishes were incubated at room temperature for 2 months and checked until mycelium or colonies appeared. All fungal endophytes were then isolated and placed in new PDA plates. After morphological and microscopic identification of all isolates, three of the most abundant endophytic fungi were retained for the rest of this study.

Molecular characterization of endophytic fungi

Genomic DNA was extracted from pure mycelia using the Universal Pathogen Kit (Omega Biotek Mag-Bind) and sequenced using the ribosomal internal transcribed spacer (ITS). PCR amplification of the fungal genomic ITS region 1 and 2 was performed using the following primers: ITS1- Forward primer (TCCGTAGGTGAACCTGCGG) and ITS4- Reverse primer (TCCTCCGCTTATTGATATGC) according to White *et al.* (8). The sequences were deposited in the NCBI database and the following accession numbers were obtained: MK880626 (*Alternaria sorghi*), MK880625 (*Alternaria tenuissima*) and MK880627 (*Alternaria alternata*).

Fermentation and extraction of endophytic fungi

Endophytic fungi were cultured on PDA at 28°C for 6 days. Subsequently, each fungus was cultivated by placing agar discs containing an actively growing pure culture of fungi isolates (5 mm in diameter) in a 250 mL Erlenmeyer flask containing 100 mL of potato dextrose broth (PDB)(9).

Each flask was incubated at 28°C for 4 weeks with periodical shaking and was examined for possible contamination. After incubation, the fermented broth was filtered through three layers of muslin cloth to remove the mycelia, then centrifuged at 5000 g for 10 min at 4°C. The supernatant was frozen and lyophilized or extracted with an equal volume of ethyl acetate. Then, the organic phase was collected and the solvent was removed by evaporation under reduced pressure at 40°C using a rotary vacuum evaporator. The samples were stored at -20°C until used.

Determination of antibacterial activity

Bacterial strains

The antibacterial activity of *Urtica dioica* leaves and endophytic fungi extracts were evaluated against reference and isolated strains. All reference strains were obtained from the Laboratory for Food Safety (Maisons-Alfort, France). The isolated strains from raw milk and acidified milk were obtained by Dr. Titouche, University of Tizi-Ouzou, Algeria (Table 1).

Five Gram-positive bacteria species including *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923 and Methicillin-Resistant *Staphylococcus aureus* (MRSA): *S. aureus* ATCC 43300, *S. aureus* LGA 251, *S. aureus* MU 50 and three Gram-negative bacteria species including *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 4352 and *Citobacter freundii* ATCC 8090 were used.

Agar diffusion method (ADM)

The lyophilized crude extracts of nettle leaves and endophytic fungi were diluted in distilled water (5 mg.mL^{-1}) and the ethyl acetate extracts of both were diluted in dimethylsulfoxide (5 mg. mL^{-1}) (DMSO, diluted to 1/10). Initially, the bacterial strains were inoculated on a sterile Muller-Hinton agar plate and incubated at 37°C for 18 h. For each extract, a sterilized 5 mm diameter disc was saturated with 20µL of crude extract and placed on the Muller-Hinton agar plates containing bacteria with turbidity adjusted to 0.5 Mc Farland in sterile physiological water. Each experiment was carried out in triplicate.

A filter paper disc saturated only with DMSO or distilled water was used as a negative control. Moreover, chloramphenicol disc was used as a positive control (30 µg/disc). All plates were incubated at 37°C for 18 h. Antibacterial activity was evaluated by measuring the diameters of the growth inhibition zones (mm) of the tested pathogenic bacteria against standards (10).

Minimum inhibitory and minimum bactericidal concentrations (MIC/MBC)

The MIC of extracts was determined using the serial macro-dilution method in Mueller-Hinton broth (11). The concentrated crude extracts were diluted using the two-fold serial dilution method. Each crude extract was diluted in series at 0.19; 0.39; 0.78; 1.56; 3.12; 6.25; 12.50 and 25 mg.mL^{-1}. The inoculates were prepared in the same medium at a density adjusted to a 0.5 Mc Farland turbidity standard (10^8 colony-forming units (CFU)/mL).

Then, 1 mL of each dilution was added to 1mL of inoculates. Culture medium without samples and other

Table 1. Phenotypic and genotypic characterization of *Staphylococcusaureus* isolates.

Strain	Sample type	*Spa-type*	ST	Toxin genes profile	Phenotype of resistance	Antimicrobial resistance genes
S84	Rawmilk	t024	8	*seb*, *sep*	OFX-FOX-TE-NOR-P	*tet* (K), *blaZ*, *mecA*
S95/S96	Acidifiedmilk	t024	8	*seb*, *sep*	OFX-FOX-TE-OX-NOR-P	*tet* (K), *blaZ*, *mecA*
S100/S101	Rawmilk	t024	8	*seb*, *sep*	OFX-FOX-TE-OX-NOR-P	*tet* (K), *blaZ*, *mecA*

OFX: ofloxacin; Fox: cefoxitin; TE: tetracycline; OX: oxacillin; NOR: norfloxacin; P: penicillin.

ST: sequence-type; *Spa*-type: staphylococcal protein A-type; *tet* (k): gene coding for resistance to tetracycline; *bla*Z: gene coding for resistance to penicillin; *mec*A: gene coding for methicillin resistance.

media without microorganisms were used as controls in the tests. The tubes were incubated at 37°C for 24 h. Bacterial growth was detected by optical density (OD) at 620 nm. The MIC value was defined as the lowest concentration of extracts preparation in which no bacterial growth occurred and which is expressed in $mg.mL^{-1}$. All tests were repeated three times and confirmed.

The MBC examination consisted of transferring 100 µL of bacterial culture from each well where no growth was observed into the Müller–Hinton Agar plates. The plates were incubated at 37°C for 24 h. Colony growth on the plates was verified after this period. MBC was defined as the lowest concentration of extract which resulted in complete inhibition of bacterial growth and is expressed in $mg.mL^{-1}$.

Determination of antioxidant activity

DPPH° radical scavenging activity

The total antioxidant activity of crude extracts was measured by DPPH (1,1diphenyl 2-picrylhydrazyl) radical scavenging capacity according to Brand-Williams *et al.* (12) with modifications. The concentration of leaves and endophytic fungi extracts were diluted in series between 50 and 450 $\mu g.mL^{-1}$. Ascorbic acid was used as a positive control. For the negative control and the blank, DPPH solution in methanol and methanol was used respectively. DPPH methanol solutions (0.1 mM, 1.95 mL) were mixed with 50 µL of extracts. The reaction mixture was vortexed and then incubated in dark conditions for 30 min at room temperature. Absorbance was measured using a spectrophotometer at 517 nm. The decrease in the absorbance of the reaction mixture indicated higher free radical scavenging activity, which was calculated using the following equation: $DPPH\ scavenging\ capacity\ (\%) = (A_0 - A_1)/A_0 \times 100\%$ where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract.

The antioxidant activity of all extracts was expressed as IC50 value ($\mu g.mL^{-1}$) (Inhibition concentration 50) calculated by linear regression analysis.

Total thiol content

Total thiol assay was performed according to Issawi *et al.* (13). Frozen samples of nettle leaves and endophytic were ground in liquid nitrogen and then extracted into 1mL of 0.2N HCl. After centrifugation at 16000 g for 4 min, 500 µL of extracted solution were neutralized with 400 µL NaOH (0.2M) and 50 µL NaH_2PO_4 (0.2M); 0.7mL of 0.12M NaH_2PO_4 (pH 7.5), 6µL of 1M EDTA, 0.1mL of 6mM Dithiobis 2-nitro benzoic acid (DTNB) were added to 0.2 mL extract. For the standards, the extract was replaced by 0; 10; 20; 50 and 100 nmol of reduced glutathione (total volume 1mL). The absorbance at 412 nm was read 5min after the addition of standards or extract.

Determination of CAT and SOD enzymatic activities

Frozen leaves and endophytic fungi samples were ground to a fine powder in liquid nitrogen (the endophytic fungi were initially cultured in PDA at 28°C for 6 days). Proteins were solubilized into 1mL of extraction buffer containing 50mM phosphate buffer pH 7.8, 1mM EDTA, 1% (w/v) polyvinylpyrrolidone and 10% (v/v) glycerol and centrifuged at 16000g for 10 min at 4° C. The supernatant was collected and used for protein quantification according to Bradford (14) and then for the assays of enzymatic activities.

Catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1) activities were assayed according to Issawi *et al.*(13). 40µg of total soluble proteins from each sample were diluted in 50 mM phosphate potassium buffer (pH 6.5). CAT activity was computed by calculating the amount of H_2O_2 with a molar extinction coefficient of $43.6M^{-1}\ cm^{-1}$. SOD activity was estimated by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by SOD. One unit of enzyme activity was taken as that amount of enzyme required to inhibit 50% of the NBT reduction.

Statistical analysis

All biological experiments were performed at least three times independently. Results were expressed as mean ± SD. Data were analyzed by Student's t-test and one-way ANOVA using the PAST free software.

Results

Isolation and molecular identification of endophytic fungi

144 isolates were obtained from nettle leaves with a colonization percentage of 18%, calculated as follows: the number of colonized segments on the total number of segments.

Three isolates were selected for this study because they were considered to be the most abundant after the morphological and microscopic characterization of the different isolates. The molecular identification of the three selected isolates was done by sequencing their ITS regions.

The result of identification showed that all isolates belong to *Alternaria* genus. Isolate 1 was closely related to *A. sorghi*, isolate 2 was closely related to *A. tenuissima* and isolate 3 was closely related to *A. alternata* with 99% or more similarity.

Antibacterial activity

The antibacterial activity was evaluated in both water and ethyl acetate extracts of *U. dioica* leaves and the three selected endophytic fungi against isolated and reference strains. All extracts displayed significant antibacterial activity against the tested bacteria in comparison with the positive control (chloramphenicol) (Table 2).

For water extract, *U. dioica* leaves exhibited similar activities toendophytic fungi with the inhibition zones ranging between 9.5 to 15.5 mm against Gram-positive bacteria (Table 2). The maximum inhibition zones were observed against the MRSA strain: MU50 and S.95, with the values of 15.5 and 14 mm respectively. *A. sorghi* exhibited the highest antimicrobial activity comparedto the other fungus.

E. coli 25922 was resistant to leaves extract. However, *Alternaria sorghi* presented the highest inhibition zone against this strain with 12.5 mm (Table 2). Overall, except *Alternaria sorghi* extract, the antibacterial activity of all extracts against Gram-positive bacteria was more effective than that observed against Gram-negative bacteria.

Table 2. Antibacterial activity of endophytic fungi and nettle leaves (water and ethyl acetate extracts), evaluated by agar diffusion method.

	Inhibition zone (mm)							
	Nettleleaves		*A. alternata*		*A. tenuissima*		*A. sorghi*	
Bacterial strains	WE	EAE	WE	EAE	WE	EAE	WE	EAE
S. aureus 25923	9.5±0.5	9.5±0.5	11.83±1.60	10.83±0.28	12.5±0.86	12.16±0.76	11.83±0.76	12.83±0.28
S. aureus 43300	10±0.5	9.16±0.28	8.16±1.25	8.16±0.28	10.33±0.57	10.16±0.28	13.16±0.76	11±0.5
S. aureus MU50	15.5±0.5	12.16±0.28	9±1	11.5±0.5	8.66±2.08	11.5±0.86	11.16±0.28	10.66±1.04
S. aureus LGA251	11.16±0.28	11±0.5	9.5±0.5	6.5±0.5	8.16±1.04	10.83±0.28	12.33±0.76	12±0.5
S. aureus S84	12.83±0.76	10±0.5	10.16±0.28	10±0.5	8.5±0.86	9.5±0.5	12.33±0.57	12.16±0.28
S. aureus S95	14±0.5	10.83±0.28	10.5±0.5	8.16±0.28	8.33±0.57	10.83±0.76	11.66±1.44	12.5±0.5
S. aureus S96	11.33±0.28	9.66±0.57	10.5±0.5	9±0.5	9.33±1.52	9.16±1.04	11±0.86	10.16±1.25
S. aureus S100	11.5±0.5	10.33±0.76	9.33±0.57	6.16±0.28	8.33±1.15	9.5±1.32	10.33±1.04	10.5±1.32
S. aureus S101	12.66±0.57	10.66±0.28	9.83±0.28	7.83±1	10.83±0.76	9.16±1.04	12±0.5	8.83±0.76
E. coli 25922	-	9.5±0.86	9±0.28	9.83±0.76	7.83±1.75	10.16±1.25	12.5±0.86	10.16±0.28
K. pneumonia 4352	8±0.5	-	6.25±0.35	8±0.5	7.25±0.35	8.83±0.76	11.16±0.76	7.83±1.32
B. cereus 10876	9.5±0.5	-	8.75±0.35	7.16±1.04	9.83±1.6	10.83±0.28	11±0.86	11.83±0.28
C. freundii 8090	7.83±0.28	9.83±0.28	8.66±0.57	7.5±0.5	9.5±1.32	9.5±0.86	11.66±0.28	8.83±0.76
Chloramphenicol (30 µg/disc)	29.23							
Distilled water	-							
DMSO(diluted 1/10)	-							

WE: Water Extracts, EAE: Ethyl Acetate Extracts, S: *Staphylococcus*, E: *Escherichia*, K: *Klebsiella*, B: *Bacillus*, C: *Citobacter*, DMSO: dimethylsulfoxide, (-): no inhibition. Results are the mean of three independent experiments ± SD.

Table 3. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations (mg.mL^{-1}) values of endophytic fungi and nettle leaves (water and ethyl acetate extracts) determined by the macrodilution method.

	Nettle leaves				*A. alternata*				*A. tenuissima*				*A. sorghi*			
	WE		EAE		WE		EAE		WE		EAE		WE		EAE	
Bacterial strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus 25923	3.12	12.50	3.12	12.50	0.78	3.12	1.56	6.25	0.39	0.78	0.78	0.78	0.78	1.56	0.39	0.39
S. aureus 43300	3.12	12.50	3.12	25	3.12	25	3.12	25	1.56	3.12	1.56	6.25	0.39	0.39	0.78	1.56
S. aureus MU50	0.19	0.39	1.56	1.56	3.12	12.50	1.56	3.12	3.12	12.50	1.56	3.12	0.78	1.56	1.56	3.12
S. aureus LGA251	1.56	3.12	1.56	3.12	3.12	12.50	Na	na	6.25	25	1.56	6.25	0.78	0.78	0.78	1.56
S. aureus 84	0.78	1.56	3.12	12.50	1.56	3.12	3.12	6.25	3.12	12.50	3.12	25	0.78	1.56	0.78	0.78
S. aureus 95	0.39	0.39	3.12	6.25	1.56	6.25	6.25	>25	3.12	25	1.56	12.50	0.78	1.56	0.39	1.56
S. aureus 96	1.56	6.25	6.25	>25	1.56	3.12	3.12	25	3.12	>25	3.12	25	0.78	3.12	1.56	6.25
S. aureus 100	1.56	3.12	3.12	12.50	3.12	25	Na	na	6.25	25	3.12	>25	1.56	6.25	3.12	12.50
S. aureus 101	0.78	1.56	3.12	6.25	3.12	25	6.25	25	1.56	3.12	3.12	>25	0.78	1.56	3.12	25
E. coli 25922	na	na	6.25	>25	3.12	12.50	3.12	6.25	6.25	>25	3.12	25	0.78	1.56	1.56	12.50
K. pneumonia 4352	6.25	>25	na	na	na	na	6.25	>25	6.25	>25	6.25	>25	1.56	12.50	6.25	>25
B. cereus 10876	3.12	25	na	na	6.25	>25	6.25	>25	3.12	12.5	1.56	3.12	1.56	6.25	1.56	3.12
C. freundii 8090	6.25	>25	6.25	25	6.25	>25	6.25	>25	3.12	25	3.12	6.25	0.78	6.25	3.12	>25

WE: Water Extracts, EAE: Ethyl Acetate Extracts, S: *Staphylococcus*, E: *Escherichia*, K: *Klebsiella*, B: *Bacillus*, C: *Citobacter*, DMSO: dimethylsulfoxide, na: not applicable.

For ethyl acetate extract, no activity was exhibited by the leaves extract against *K. pneumonia* 4352 and *B. cereus* 10876, while this extract exhibited stronger activity against the MRSA strain *S. aureus* MU50 with 12.16 mm (Table 2). *A. sorghi* generally displayed stronger antimicrobial activity toward bacterial tested than the other fungi, except for the MRSA strain *S. aureus* MU50. Furthermore, the ethyl acetate extracts had a weak inhibitory effect on Gram-negative strains.

MIC and MBC values of leaves and endophytic fungi preparations, water and ethyl acetate extracts were also determined as an evaluation of their activity against selected bacteria (Table 3). For the antibacterial evaluation, all extracts were able to inhibit the growth of Gram-positive and Gram-negative bacteria, with MIC and MBC values ranging from 0.19 to 25 mg.mL^{-1}. Both extracts of *U. dioica* leaves and endophytic fungi had bacteriostatic and bactericidal activity against all examined bacteria (Table 3).

For water extract, leaves exhibited the highest activity against *S. aureus* MU50 with bactericidal activity with MIC and MBC values of 0.19 and 0.39 mg.mL^{-1} respectively (Table 3). Bactericidal activity against isolated *S. aureus* with MIC and MBC values ranging from 0.39 to 1.56 mg.mL^{-1}respectively,was also examined. *A. sorghi*extract showed the bactericidal activity against all bacteria tested with MIC and MBC values ranging-between 0.39 and 3.12 mg.mL^{-1}.

For ethyl acetate extract, *A. sorghi* exhibited the highest antibacterial activities against reference and isolated *S. aureus* strains with MIC and MBC values ranged between 0.39 to 1.56 mg.mL^{-1} (Table 3).

DPPH free radical scavenging activity

Antioxidant activity monitored as DPPH radical scavenging activity showed that ethyl acetate extract presented the highest antioxidant activity compared to the water extract since its radical scavenging capacity was similar to that of ascorbic acid (Fig. 1).

For water extracts, *U. dioica* leaves extract had

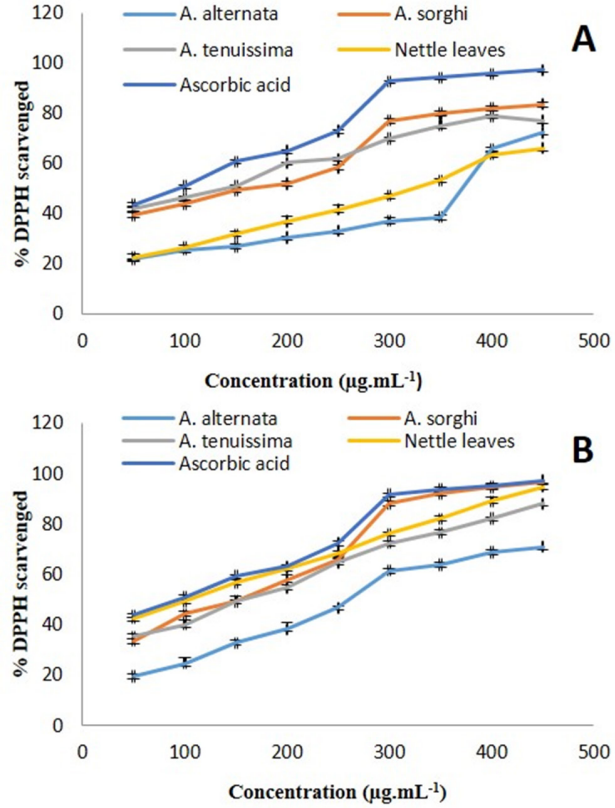

Figure 1. DPPH radical scavenging activities of endophytic fungi and nettle leaves water extracts (A) and ethyl acetate extracts (B) in comparison with ascorbic acid (results are the mean of three independent experiments ± SD, for all P <0.001).

the lower percentage inhibition of DPPH radical than all fungal extracts excepted for *A. alternata*. The free radical scavenging activities of the endophytic fungi extracts were as following: *A. alternata* (72.10 ± 0.60 %), *A. tenuissima* (76.77 ± 0.84%) and *A. sorghi* (83.53 ± 0.70 %) at 450 µg.mL^{-1} extract. All extracts showed significant difference ($p < 0.001$) when compared with ascorbic acid (97.30 ± 1.05 %).

For the ethyl acetate extracts, the nettle leaves and *A. sorghi* exhibited the greatest antioxidant activity with DPPH free radical scavenging values of 94.87 ± 1.48 % and 96.88 ± 1.15 %, respectively, while *A. tenuissima* and *A.alternata* gave 87.98 ± 0.64 % and 70 ± 1.23%, respectively. The low IC50 value indicated a high antioxidant activity, thus indicating higher antioxidant activity of the nettle leaves (104.63± 3.33 µg.mL^{-1}) than *A. sorghi* (139.27±1.56 µg.mL^{-1}).

Total thiol content and ROS detoxification enzymes

This study also focused on the quantification of total thiol compounds, considered as principal antioxidant molecules and investigated ROS detoxification enzyme activities such as catalases and superoxide dismutases, to assess the potential role of endophytic fungi in the regulation of abiotic and biotic stresses in the plant. Therefore, these enzymes and compounds were measured in nettle leaves and a 6-day old mycelium.

Unexpectedly, all fungi showed nearly the same amount of total phenolic compounds as nettle leaves. However, this was not the case for total thiols; hence, nettle leaves exhibited the highest level as the content of thiols was approximately two-fold more than that of fungi (Fig. 2).

Surprisingly, catalase demonstrated no significant variation between nettle leaves and all fungi. Nevertheless, nettle leaves which had the same SOD activity as *Alternaria alternata* showed significantly increased SOD activity than *Alternaria tenuissima* as well as *Alternaria sorghi* (Fig. 3).

Discussion

The antibacterial activities of the leaves of *Urtica dioica* and its three endophytic fungi were carried out using two types of extraction: water and ethyl acetate.

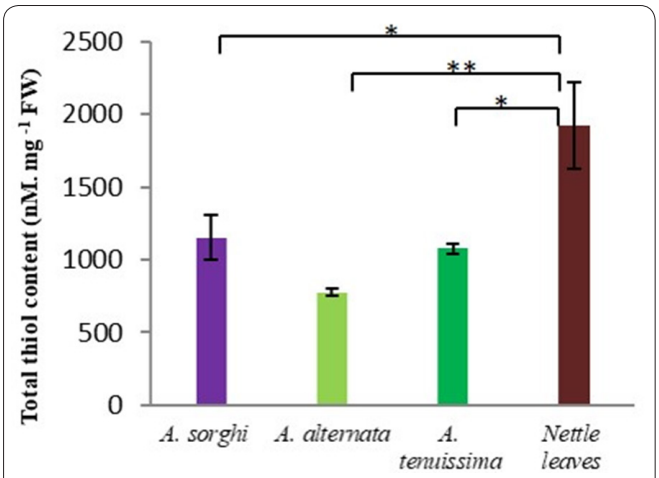

Figure 2. Total thiol contents in nettle leaves and its endophytic fungi (FW:fresh weight, results are the mean of three independent experiments ± SD, **P <0.01; *P <0.05, ns: not significant).

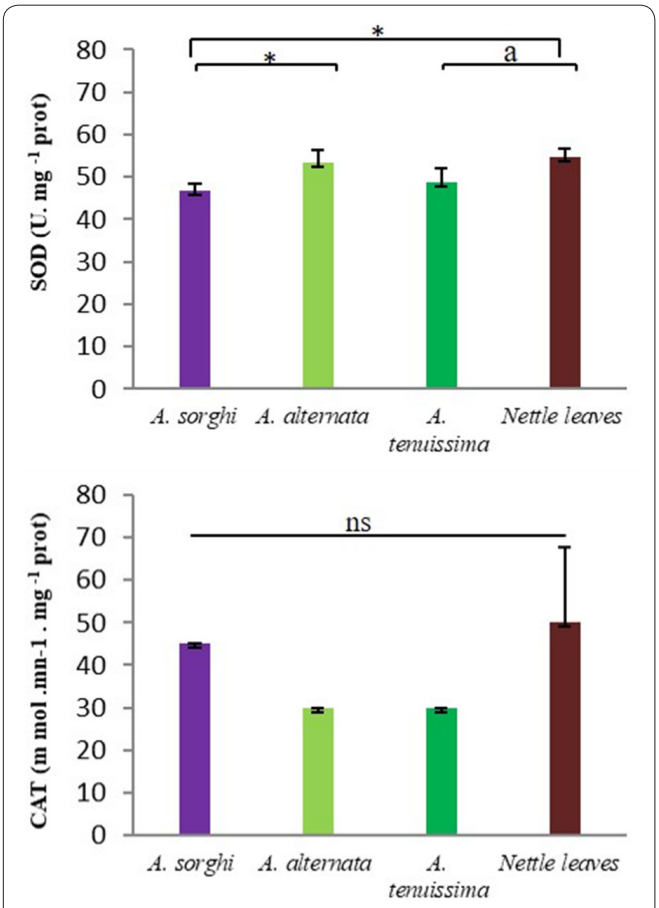

Figure 3. ROS detoxification enzymes specific activities in Nettle leaves and its endophytic fungi, CAT: catalase, SOD:superoxide dismutase (results are the mean of three independent experiments ± SD, *P <0.05, a: P<0.1, ns: not significant).

This work is reported to be the first of its kind to focus on foliar endophytic fungi of *Urtica dioica*, however, many studies report the predominance of *Alternaria* in different host plants (3, 15). It is true that *Alternaria* species have been described as plant pathogenic fungi, referred to as saprophytes or plant parasites. However, *Alternaria sp*, has been recorded as endophytic fungi colonizing several host plants including medicinal plants (16–19). In addition, Tsai *et al* (20) showed that endophytic fungi have pathogenic fungi as their ancestors, which have a very different life cycle.

All extracts have interesting inhibition zones against the majority of the bacteria tested, but they remain less than those obtained by the positive control, chloramphenicol. The abundance of molecules of all kinds in the extracts and the interactions that result from them could undoubtedly explain this difference. Gram-positive bacteria are more sensitive to extracts than Gram-negative bacteria. This difference could be attributed to the difference in membrane composition, Gram-negative bacteria, unlike Gram-positive bacteria, have a phospholipid outer membrane containing lipopolysaccharide as a major structural component, making the cell wall more impermeable to antibacterial agents (21).

The results showed that *E. coli* ATCC 25922 strain was resistant to water extract from *Urtica dioica* leaves. Gülcin *et al*. (22) showed low bacterial activity of water extracts of the same species with an inhibition zone of 8 mm while those of Salinas-Sánchez *et al*. (23) reported the inefficiency of ethanol extracts from nettle leaves against *E. coli A*TCC 9837 strain. The ethyl acetate extract of nettle leaves was also inactivated against *K. pneumonia* ATCC 4352. This result is consistent with the results reported by Körpe *et al*. (24), where extracts of *U. dioica* and *U. pilulifera* leaves showed no activity against *K. pneumonia*. However, we showed that the water extract of *A. sorghi* showed an interesting activity against *E. coli* ATCC 25922.

Endophytic fungi and especially *Alternaria sp.* can produce new bioactive compounds such as paclitaxel, podophyllotoxin and camptothecin (25). Moreover, *Aspergillus parasiticus* could transform α-santonin into 3,4-epoxy- ^{L}α- santonin, with high antibacterial activity (26).

A satisfactory antibacterial activity was also observed during this analysis for the extracts studied against reference MRSA strains or isolated from raw or acidified milk. The water extracts showed the most important activity against *S. aureus* MU50 and *S. aureus* S95 strains with inhibition zones of 15.5 and 14.05 mm respectively. The water extract of *A. sorghi* showed bactericidal activity against strains of *S. aureus* ATCC 43300 (CMI 0.39 mg.mL^{-1}; CMB 0.39 mg.mL^{-1}) and *S. aureus* LGA 251 (MIC 0.78 mg.mL^{-1}; CMB 0.78 mg.mL^{-1}). These results are in good agreement with Gülcin *et al*. (22) and Tasdelen Fisgin *et al*. (27) studies and are particularly interesting as clinical isolates of *S. aureus*, especially those resistant to Methicillin (28).

Alternaria species are known as a source of many bioactive substances with very different structures and activities (29, 30). Thus, boric acid D and Altenusin of *Alternaria sp.*, showed inhibitory activity against *Staphylococcus aureus* with CMI of 100µg.mL^{-1} and 25µg.mL^{-1}, respectively (31). In addition, some anthraquinones of *A. solani* and *Alternaria sp.* showed antibacterial activity on *Bacillus subtilis, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus albus, Staphylococcus aureus an*d *Vibrio parahemolyticus* (32–34).

Pyrone derivatives of the endophyte fungus *A. tenuissima* of the Chinese medicinal plant, *Salvia przewalskii*, also exhibited antibacterial activity against the following strains: *B. subtilis*, *B. megaterium* and *C. perfringen* (35). Sonaimuthu *et al*. (4) demonstrated that tenuazonic acid is an active compound of *A. alternata* against *Mycobacterium tuberculosis* H37Rv (CMI 250µg.mL^{-1}).

In this study, the total antioxidant activity of water and ethyl acetate extracts of *A.* endophytes and their host revealed that in general, all the extracts studied have high antioxidant activity. First of all, we performed a DPPH assay that is based on two scavenging mechanisms: electron transfer (ET) and hydrogen transfer (HAT) thus, this assay reflected the typical and global antioxidant capacity (36). Our results showed that the greatest free radical scavenging activity of DPPH was observed with ethyl acetate extracts and particularly those of *A. sorghi* and nettle leaves. Thus, ethyl acetate extracts from the leaves of *U. dioica* and *A. sorghi* exhibited the highest anti-free radical activity with a DPPH trapping rate of 94.86% and 96.87%, respectively. Such results were consistent with extracts of *A. sorghi* and nettle leaves rich in phenolic compounds that are extracted by polar solvents as ethyl ether (data not shown). Our results indicated that nettle leaves and endophytic fungi are an important source of total thiols. As it has been shown that alterations in cell thiol content are the main determinant of changes in the total antioxidant capacity of the cell, which would make it a useful parameter for the evaluation of oxidative stress (37). Thus, the highest rates of total thiols in the nettle leaves indicated that endophytic fungi helped their hosts to increase thiol antioxidants conferring them to withstand biotic and abiotic stresses (38).

This work also focused on ROS detoxification enzymes, including catalase (CAT) and superoxide dismutase (SOD). It is well known that in plants, catalase eliminates H_2O_2 generated during photorespiration and fatty acid oxidation, it also plays an important role in detoxifying active oxygen species generated by various types of environmental stress (39). Superoxide dismutase catalyses the dismutation of the superoxide anion ($O_2^{\bullet-}$) into O_2 and H_2O_2. The oxygen peroxide thus produced will then be eliminated by catalase and/or peroxidases (40). Results showed that while there was no significant variation in catalase activity in nettle and fungi, the SOD dismutation capacity increased markedly in *U. dioica* leaves. Indeed, SOD represents the first line of defense against superoxide anion scavenging and enzymes than catalase such as peroxidases (glutathione peroxidase, ascorbate and guaiacol peroxidases) are involved in the defense against hydrogen peroxide production (41).

However, Bonnet *et al*. (42) reported the presence of the endophyte *Epichloë lolii* modified the metabolism by promoting catalase catalysis of H_2O_2 and improving the host's defence capacity. Especially since an increase in the activity of ROS detoxification enzymes

has been shown in individuals of *E. dahuricus* exposed to drought (6).

To date, the mechanisms highlighted by endophytic fungi to improve the plant's defence systems remain unknown. Therefore, the SOD gene was isolated in *E. lolii*, which provides a new approach for studying the role of gene expression in the detoxification capacity of ROS and the interaction mechanism between endophyte and plant. Zhang *et al*. (5) showed that relative expression of the genes encoding SOD, POD and CAT and their corresponding enzymes increased in wheat seedlings under saline stress when *Trichoderma longibrachiatum* T6 strain was applied, indicating that this strain significantly protected seedlings from salt-related stress.

The study showed that *A. sorghi* presented the best activities compared to other isolated fungi, in fact, it is common for a plant to be colonized by fungi belonging to the same genus or species and only one or more biotypes of a given fungus will produce a biological actives compounds in culture, that will need further investigations to characterize.

Acknowledgments

Thankto Pr. Sol V. to have welcome us in his laboratory as well as Pr. Riba A. and Dr. Hamdouche Y. for fungal strain identification.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Strobel GA. Endophytes as sources of bioactive products. Microbes Infect2003;5:535-544.
2. Aziziaram, Z., Bilal, I., Zhong, Y., Mahmod, A., Roshandel, M. Protective effects of curcumin against naproxen-induced mitochondrial dysfunction in rat kidney tissue. Cell Mol Biomed Rep 2021; 1(1): 23-32.
3. Kumar S, Kaushik N, Edrada-Ebel R, Ebel R and Proksch P Isolation, characterization, and bioactivity of endophytic fungi of *Tylophora indica*. World J Microbiol Biotechnol2011; 27 (3): 571-577.
4. Sonaimuthu V, Parihar S, Thakur JP, Luqman S, Saikia D, Chanotiya CS, Jhonpaul M and Negi A. Tenuazonic acid: A promising antitubercular principle from *Alternaria alternata*. Microbiol Res2011; 2 (2): 63-65.
5. Zhang S, Gan Y and Xu B. Application of Plant-Growth- Promoting Fungi *Trichoderma longibrachiatum* T6 Enhances Tolerance of Wheat to Salt Stress through Improvement of Antioxidative Defense System and Gene Expression. Front Plant Sci2016; 7(1405): 1-11.
6. Zhang Y, Zhou Y, Zhang X, Duan T and Nan Z. Effects of *Epichloë* endophyte on antioxidant enzymes activities, photosynthesis and growth of three ecotypes of *Elymus dahuricus*. Front Agr Sci Eng2018; 5(1): 148-158.
7. Schulz B, WankeS, Draeger S and Aust HJ. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol Res1993;97(12): 1447-1450.
8. White TJ, Bruns T, Lee S and Taylor J Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications1990; 18: 315-22.
9. Nath A, Raghunatha P and Joshi SR Diversity and Biological Activities of Endophytic Fungi of *Emblica officinalis*, an Ethnomedicinal Plant of India. Mycobiol2012;40(1): 8-13.
10. Valgas C, Machado de Souza S, Smania EFA and Smania A. Screening methods to determine antibacterial activity of natural products. Braz J Microbiol2007;38 (2): 369-380.
11. Saha A and Rahman MS. Antimicrobial Activity of Crude Extract from *Calycopteris floribunsa*. Bang J Microbiol2008;25 (2): 137-139.
12. Brand-Williams W, Cuvelier ME and Berset C. Use of free radical method to evaluate antioxidant activity. LWT-Food Sci Technol1995; 28: 25-30.
13. Issawi M, Guillaumot D, Sol V and Riou C. Responses of an adventitious fast-growing plant to photodynamic stress: comparative study of anionic and cationic porphyrin effect on *Arabidopsis thaliana*. Physiol Plant2018; 162 (3): 379-390.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem1976; 72: 248-254.
15. Khan R, Shahaz S, Choudhary MI, Khan SA and Ahmad A. Communities of endophytic fungi in medicinal plant *Withania somnifera*. Pak J Bot2010; 42 (2): 1281-1287.
16. Aly AH, Edrada-Ebel R, Indriani ID, Wray V, Müller WE, Totzke F, Zirrgiebel U, Schächtele C, Kubbutat MH, Lin WH, ProkschP and Ebel R. Cytotoxic metabolites from the fungal endophyte *Alternariasp*. and their subsequent detection in its host plant *Polygonum senegalense*. J Nat Prod2008;71(6): 972-980.
17. Naik BS, Shashikala J and Krishnamurthy YL. Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India. Fungal Ecol2008; 1(2): 89-93.
18. Woudenberg JHC, Groenewald JZ, Binder M and Crous PW (2013) *Alternaria* redefined. Stud Mycol 75 (1): 171-212.
19. Govindappa M, Channabasava R, Sadananda TS, Chandrappa CP and Umashankar T. Identification of bioactive metabolites by GC-MS from an endophytic fungus, *Alternaria alternate* from *Tabebuia argentea* and their in vitro cytotoxic activity. Int J Biolo Pharm Res2014; 5(6):527-534.
20. Tsai HF, Liu JS, Staben C, Christensen MJ, Latch GC, Siegel MR and Schardl CL Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloe* species. Proc Natl Acad Sci USA1994;9 (7): 2542-2546.
21. Kaushik A, Aron A, Mihreteab S, Mohamedkassm N and Michael Kidane E. Phytochemical screening and antimicrobial activity of medicinal plants used by eritrean traditional healers. World J Pharm Res 2015; 4 (4): 501-509.
22. Gülcin I, Küfrevioglu OI, Oktay M and Büyükokuroglu ME. Antioxidant, Antimicrobial, Antiulcer and Analgesic Activities of Nettle (*Urtica dioica* L.).J Ethnopharmacol 2004;90(2-3): 205-215.
23. Salinas-SánchezDO, Arteaga-Najera GL, Leon-Rivera I, Dorado Ramirez O, Guadalupe Valladares CisnerosM and Navarro García VM. Antimicrobial Activity of Medicinal Plants from the Huautla Sierra Biosphere Reserve in Morelos (México). Polibotánica2009; 28: 213-225.
24. Körpe DA, İşerİ ÖD, Sahin FI, Cabi E and Haberal M. High-antibacterial activity of *Urtica spp*. Seed extracts on food and plant pathogenic bacteria International. J Food Sci Nutr 2013; 64(3): 355-362.
25. Zhao J, Shan T, Mou Y and Zhou L. Plant-derived bioactive compounds produced by endophytic fungi. Mini Rev Med Chem 2011; 11(2): 159-168.
26. Siddhardha B, Gadupudi R and Basaveswara Rao MV. Biotransformation of (-)-a-santonin by *Aspergillus parasiticus* and antimicrobial efficacy of the transformed products. Curr Biotechnol 2012; 1(3): 194-198.
27. Tasdelen Fisgin N, Tanriverdi Cayci Y, Coban AY, Ozatli D, Tanyel E, Durupinar B and Tulek N. Antimicrobial activity of plant

extract Ankaferd Blood Stopper. Fitoterapia 2009;80(1):48-50.
28. Gould IM. The clinical significance of methicillin-resistant Staphylococcus aureus. J Hosp Infect 2005; 61 (4): 277-282.
29. Liebermann B, Ellinge R and Ihn W. ACTG-toxins produced by a strain of *Altemaria altemata*. That does not originate from *Citrus* species. Phytopathology 1994;140 (4): 385-388.
30. Tan RX and Zou WX. Endophytes: a rich source of functional metabolites. Nat Prod Rep 2001;18(4): 448-459.
31. Xu X, Zhao S, Wei J, Fang N, Yin L and Sun J.Porric acid D from marine-derived fungus *Alternaria sp*. isoltated from Bohai Sea. Chem Nat Compd 2012; 47(6): 893-895.
32. Okamura N, Haraguchi H, Hashimoto K and Yagi A. Altersolanol-related antimicrobial compounds from a strain of *Alternaria solani*. Phytochemistry1993;34(4):1005-1009.
33. Yagi A, Okamura N, Haraguchi H, Abo T and Hashimoto K. Antimicrobial tetrahydroanthraquinones from a strain of *Alternaria solani*. Phytochemistry 1993;33(1): 87-91.
34. Zheng CJ, Shao CL, Guo ZY, Chen JF, Deng DS, Yang KL, Chen YY, Fu XM, She ZG, Lin YC and Wang CY. Bioactive hydroanthraquinones and anthraquinone dimers from a soft coral-derived *Alternaria sp*. fungus. J Nat Prod2012;75 (2): 189-197.
35. Wang XZ, Luo XH, Xiao J, Zhai MM, Yuan Y, Zhu Y, Crews P, Yuan CS and Wu QX. Pyrone derivatives from the endophytic fungus *Alternaria tenuissima* SP-07 of Chinese herbal medicine *Salvia przewalskii*. J Fitote2014;99: 184-190.
36. Liang N and Kitts DD. Antioxidant property of coffee components: assessment of methods that define mechanisms of action. Molecules2014; 19 (11):19180-208.
37. Balcerczyk A and Bartosz G. Thiols are Main Determinants of Total Antioxidant Capacity of Cellular Homogenates. Free Radic Res2003;37(5): 537-541.
38. Kasotia A and Choudhary DK. Role of Endophytic Microbes in Mitigation of Abiotic Stress in Plants. In: Parvaiz A and Saiema R (ed.), Emerging technologies and management of crop stress tolerance. Elsevier, San Diego, London, Waltham 2014; pp. 97-108.
39. Scandalios JG. Regulation of the antioxidant defence genes Cat and Sod of maize. In: Scandalios, JG (ed.), Molecular Biology of Free Radical Scavenging Systems. Cold Spring Harbor Laboratory Press, New York, 1992; pp. 117-152.
40. Racchi ML. Antioxidant Defenses in Plants with Attention to *Prunus* and *Citrus spp*. Antioxidants 2013; 2 (4): 340-369.
41. Dastogeer KMG, Li H, Sivasithamparam K, Jones MGK and Wylie SJ. Fungal endophytes and a virus confer drought tolerance to *Nicotiana benthamiana* plants through modulating osmolytes, antioxidant enzymes and expression of host drought responsive genes. Environ Exp Bot2018; 149: 95-108.
42. Bonnet M, Camares O and Veisseire P. Effects of zinc and influence of *Acremonium lolii* on growth parameters, chlorophyll a fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne* L. cv Apollo). J Exp Bot 2000; 51(346): 945-953.