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PHARMACOGNOSIE

In vitro evaluation of antioxidant, antibacterial and antifungal activities of *Terfezia claveryi* Chatin

Évaluation des activités antioxydante, antibactérienne et antifongique de *Terfezia claveryi* Chatin

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Abstract The present study was conducted to investigate the antioxidant and antimicrobial activities of *Terfezia claveryi* Chatin. Soxhlet apparatus was used for extraction with a series of solvents, dichloromethane, chloroform, ethyl acetate and methanol in sequence of increasing of polarity. Also maceration extraction with methanol was used. Our results revealed that the extraction methods and the nature of solvents had a significant effect on the antioxidant and antimicrobial activities.

T. claveryi extracts were investigated for their antioxidant activity. The antioxidative properties of this desert truffle were determined by radical-scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The methanolic extract showed the most potent radical-scavenging activity on DPPH radicals (IC_{50}=8.56 mg/mL). However chloroform and ethyl acetate extracts showed no antioxidant activity at the concentration of 5-40 mg/mL.

The antimicrobial activity of *T. claveryi* was evaluated using agar well diffusion methods against nine species of bacteria and one yeast. The results showed that the majority of the extracts investigated obtained by Soxhlet extraction (dichloromethane, chloroform, ethyl acetate and methanol extracts) showed greater activities against the Gram-positive bacteria, Gram-negative bacteria and the yeast compared to the macerate extract. The strongest antimicrobial activity was exhibited by the chloroform extract of *T. claveryi* which minimum inhibitory concentration values ranged from 12.5 to 100 mg/mL.

Keywords Desert truffle · *Terfezia claveryi* Chatin · Antioxidant activity · Antibacterial activity · Antifungal activity

Résumé Au cours de ce travail, nous avons évalué les activités antioxydante et antimicrobienne de *Terfezia claveryi* Chatin. L'extraction a été faite à partir des ascocarpes de *T. claveryi* dans quatre solvants organiques à polarité croissante : dichlorométhane, chloroforme, acétate d'éthyle et le méthanol à l'aide d'un appareil type Soxhlet. La macération avec du méthanol a été également utilisée. Nos résultats ont révélé que les méthodes d'extraction et la nature des solvants ont un effet significatif sur l'activité antioxydante et antimicrobienne.

Les propriétés antioxydantes des différents extraits de *T. claveryi* ont été déterminées par la capacité antiradicalaire de 1,1-diphenyl-2-picrylhydrazyl (DPPH). L'extrait méthanolique a montré une puissante activité de piégeage sur les radicaux DPPH (IC50 = 8.56 mg/mL). Cependant, les extraits chloroformique et d'acétate d'éthyle n'ont montré aucune activité antioxydante à la concentration de 5-40 mg/mL.

L'activité antimicrobienne de *T. claveryi* a été évaluée en utilisant la méthode de diffusion par puit sur gélose contre neuf espèces bactériennes et une levure. Les résultats ont montré que la majorité des extraits étudiés obtenus par Soxhlet ont montré une plus grande activité contre les bactéries Gram-positives, Gram-négatives et la levure par rapport à l'extrait obtenu par macération. L'activité antimicrobienne la plus forte a été constatée par l'extrait chloroformique de

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T. claveryi lequel les valeurs de concentration inhibitrice minimale ont varié de 12,5 à 100 mg/mL.

Mots clés Truffe du désert · *Terfezia claveryi* Chatin · Activité antioxydante · Activité antibactérienne · Activité antifongique

Introduction

The genus *Terfezia* is an edible fungi with important gastronomic, nutritional and medicinal properties, it belongs to the so-called Desert Truffles, locally named « terfess », which are a complex family of mycorrhizal hypogeous fungi. Their geographical distribution is limited to arid and semiarid lands, mostly in countries around the Mediterranean basin. *Terfezia* was shown to belong to the Pezizaceae rather than to the distinctly hypogeous Terfeziaceae family, which was therefore abolished [1,2]. Truffles are considered to be one of the oldest foods known for their nutritional value, especially when compared with meat and fish [3].

Desert truffles are a rich source of protein, amino acids, fatty acids, minerals and carbohydrates [4]. They have been used as traditional medicine in Arabia for over two millennia without known toxic harmful effects to its users [5]. As a fungal drug remedy, a boiled truffle water-extract is highly recommended by Bedouins as an eye wash for the treatment of one of the most common eye diseases at that time, e.g., trachoma [6]. This practice developed the following recommendations of the Prophet Mohammad (Peace be upon him) whom was reported to have said: "Truffles are from man (as they grow naturally without man's care) and their water is a cure for the eye".

Rougieux [7] was the first researcher to investigate the biological activity of *Terfezia boudieri* Chatin extract on bacterial growth *in vitro*. He reported that amyl acetate extract of *T. boudieri* had a low inhibition potency against *Staphylococcus aureus*.

Al-Marzooky [8] has tested the biological activity of desert truffle extracts on bacterial growth *in vitro*. He reported that all aqueous, polar and non-polar extracts of *Terfezia claveryi* exhibited good antibacterial activity against broad spectrum of the tested bacterial species, in particular, the causal organism of trachoma *Chlamydia trachomatis*.

Chellal and Lukasova [9] have reported that antibiotics extracted from the desert truffles *Terfezia* and *Tirmania* spp. proved effective against bacteria. The most important bacteriostatic activity against Gram (+) bacteria including *Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus* was obtained with methanolic extract at 45°C from *Terfezia*. Finally, they noticed that extracts of *Tirmania* were less bacteriostatic than those of *Terfezia*.

Janakat et al. [10] have studied the antimicrobial activity of aqueous and methanolic extracts from *Terfezia claveryi* against *Staphylococcus aureus in vitro*. They showed that 5% of aqueous extract inhibited the growth of *S. aureus* by 66.4%. However, the methanolic extract did not cause a significant growth inhibition. In another study, Janakat et al. [11] have investigated the antimicrobial activity of aqueous and methanolic extracts from *Terfezia claveryi* against *Pseudomonas aeruginosa* in vitro. 5% of aqueous extract inhibited the growth of *P. aeruginosa* by 40.9%, while methanolic extract was ineffective.

Moreover, Dib-bellahouel and Fortas [12], have tested two fractions of ethyl acetate extract of *Tirmania pinoyi* (Maire) against the bacteria *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 14028 and *Enterococcus* sp. ATCC 29212. The results showed inhibition zones, with diameters between10 and 22 mm, against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538. The GC-MS showed the presence of several products, some of them have antibacterial activity in the literature.

Furthermore, Neggaz and Fortas [13] have studied the antimicrobial activity using disc agar method of six fractions which were separated from the ethyl acetate extract of *Tirmania pinoyi* (Maire) ascocarps using two chromatographic methods. The results showed that only two fractions (F2 and F6) inhibited growth of both Gram negative (*Pseudomonas aeruginosa* ATCC14028, *Escherichia coli* ATCC25922) and Gram positive bacteria (*Staphylococcus aureus* ATCC6538, *Enterococcus faecalis* ATCC2035) and had significant antifungal activity against *Candida albicans* ATCC10231.

In another study, Aldebasi et al. [14] have reported that *Terfezia claveryi* aqueous extract exhibited excellent antibacterial activity against all clinical isolates of corneal ulcer tested, especially against *Pseudomonas aeruginosa* which showed the maximum antibacterial activity with mean zone of inhibition 20.33 mm at concentration of 100 mg/mL.

Recently, Hamza et al. [15] have tested the truffle extracts for their antibacterial activity against seven species of bacteria three Gram-negative (*Salmonella typhimurium* (NRRLB4420), *Escherichia coli* (ATCC19115) and *Pseudomonas aeruginosa* (ATCC27853)) and four Gram-positive (*Enterococcus faecalis* (ATCC29212), *Staphlyococcus aureus* (ATCC25923), *Staphlyococcus epidermidis* (CIP106510) and *Bacillus subtilis* (ATCC168)). The methanolic extract also exhibited remarkable inhibitory activity on the tested strains, which minimum inhibitory concentration values ranged from 0.25 to 1.3 mg/mL. The results showed also that the methanolic extract displayed the highest DPPH radical-scavenging activity (IC_{50}= 0.20 mg/mL).

Additionally, Dogan et al [16] have studied the antimicrobial activity of chloroform, acetone and methanol extracts

of *Terfezia boudieri* against four Gram-positive (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6633, *Listeria monocytogenes* type2 NCTC 5348 and *Streptococcus pyogenes* ATCC 19615) and five Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Klebsiella pneumonia* ATCC 10031, *Pseudomonas aeruginosa* ATCC 15442, *Proteus vulgaris* ATCC 7829 and *Salmonella enteritidis* RSHMB), and one yeast *Candida albicans* ATCC 1023. In this study, the lowest minimum inhibitory concentration (MIC) value was observed with the acetone extract (MIC 4.8 µg/mL) against *C. albicans*. Maximum antimicrobial effect was also determined with the acetone extract (MIC 4.8-312.5 µg/mL). The scavenging effect of *T. boudieri* on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals was measured as 0.031 mg/mL at 5 mg/mL concentration, and its reducing power was 0.214 mg/mL at 0.4 mg/mL. The results showed that *T. boudieri* has antimicrobial activity on the Gram negative and positive bacteria as well as yeast, and it also has a high antioxidant capacity.

Traditionnel uses of desert truffles have motivated our effort to investigate the antioxidant and antimicrobial activities. Therfore, the aim of this study is to evaluate the antioxidant, antibacterial and antifungal activities of different extrats of *T. claveryi*.

Materials and methods

Truffle materials

Fresh *Terfezia claveryi* Chatin ascocarps, with no apparent physical or microbial damage, were purchased from local markets in Tiaret (Algeria) and were authenticated by Pr. FORTAS, Laboratory of Biology of Microorganisms and Biotechnology (LBMB), Faculty of Life Sciences and Nature, University of Oran1 Ahmed Benbella, Algeria. All the truffle samples were identically selected in terms of size, shape, colour, and ripening stage. The fresh truffles were cleaned from soil and dried in the shadow, they were then ground into fine powder and stored at ambient temperature in a dry place and in the dark until use.

Extraction procedures

Soxhlet extraction

The dried truffle powder of *T. claveryi* (100 g) was successively extracted by Soxhlet apparatus using solvents of increasing polarity as follows: dichloromethane, followed by chloroform, ethyl acetate and finally with methanol during 24 h for each solvent. The volume of each solvent used is 500 mL which was then evaporated under vacuum using a rotary evaporator and then weighed to calculate the yield of the extracts and stored at 4°C until required.

Maceration extraction with methanol

10 g of *T. claveryi* powder and 100 mL of methanol are introduced into an Erlenmeyer flask. The whole is macerated for 24 h at room temperature. The extract was filtered through Whatman No.1 filter paper and the filtrate was collected; then the methanol of the crude extract was removed using a rotary evaporator. Finally, the obtained extract was kept in the dark at +4°C until further analysis.

Assay of DPPH scavenging activity

The DPPH radical scavenging activity was determined by a spectrophotometric method based on the reduction of methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). 50 µL of each extract at different concentrations (5, 15, 25 and 40 mg/mL) was added to 2 mL methanolic solution of DPPH (0.2 mM). The mixture was allowed to react at room temperature in the dark for 30 min. α-tocopherol is used as positive control. After 30 min, the absorbance (A) was measured at 517 nm. The experiment was repeated for three times for each test sample [17].

IC_{50} values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals. DPPH free radical-scavenging activity was calculated according to the following equation:

DPPH radical-scavenging activity (%) = (Abs control – Abs sample) / (Abs control) × 100 where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract / standard

Antimicrobial assay

Microbial strains

Microbial cultures of ten different strains of both Gram positive, Gram negative bacteria and yeast were used for the determination of antimicrobial activity. Four Gram-positive *(Staphylococcus aureus ATCC 6538, Enterococcus feacalis ATCC 2035, Bacillus subtilis ATCC 6633* and *Corynebacterium)*, five Gram-negative *(Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 14028, Klebsiella pneumoniae ATCC 4352, Proteus vulgaris* and *Salmonella typhi)* and one yeast species (*Candida albicans ATCC 10231*).All tested strains were collected from the culture collection of Pasteur Institute of Algeria (IPA).

Inoculum preparation

The bacterial strains were grown overnight at 37°C in Nutrient Agar, while *C. albicans* was grown in Saboraud Agar. Inoculum for the assays was prepared by inoculating three to five colonies from the agar plate culture into 10 mL of nutrient broth and then incubated at 37°C for 24h. After growing, the microbial suspension was standardized with sterile saline to turbidity equivalent to 0.5 McFarland scale (10^8 CFU/mL for bacteria and 10^6 CFU/mL for *C. albicans*).

Determination of antimicrobial activity

The antimicrobial activity of *T. claveryi* extracts was determined using agar well diffusion method following published procedure with slight modification [18].

Antibacterial activity

Each bacterial suspension was spreaded over the surface of Muller-Hinton Agar plates. The plates containing wells of 6 mm diameter were filled with 30 µL extracts. The plates were then incubated at 37°C for 24 h. The results were expressed in terms of the diameter of the inhibition zone. All tests were performed in triplicates. Streptomycin (30 µg) was used as positive control, while solvants were used as a negative control against bacteria and yeast, respectively.

Antifungal assay

C. albicans was cultured in Sabouraud Broth for 48 h at 27°C and Sabouraud Agar was employed for the agar well diffusion experiments as explained above. Fungal suspensions were adjusted to 10^5 cells/mL. Inhibition zones were determined after incubation for 48 h at 27°C. All tests were performed in triplicates.

Determination of minimum inhibitory concentration (MIC)

The MIC of the *T. claveryi* extracts were determined by a macrodilution broth method [18]. Serial dilutions were prepared in macrodilution tubes with concentrations ranging between (6.25-100 mg/mL).

10 µL of microbial suspension at the final density of 0.5 McFarland were added to all tubes. After 24 h incubation at 37°C for bacteria and 30°C for yeast, the inhibition of microbial growth was evaluated by measuring the turbidity of each tube and compared to the control. The lowest concentration that produced an inhibitory effect was recorded as the MIC for each extract.

Determination of minimal bactericidal concentration (MBC)

The MBC of the *T. claveryi* extracts were determined by a macro broth dilution method [18]. MBC values were determined taking into account the MIC values. 25 µL taken from tubes without microbial growth (including MIC), and inoculated on Muller Hinton agar plates. After 24 h incubation at 37 °C for bacteria and 30°C for yeast, the lowest concentration which did not show any macroscopic growth of tested microorganism was identified as the MBC.

Statistical analysis

Excel (Microsoft Corporation, USA) and Statistica (version 7.1) were used for statistical analysis. Data are expressed as means ± SD. To assess the variation of the variables among samples, a two-way Analysis Of Variance (ANOVA) was performed. Statistical significance between means was determined using Duncan's multiple range tests and set at $p < 0.05$.

Results

The yields of dichloromethane, chloroform, ethyl acetate and methanol of *T. claveryi* extracts were 7%, 1%, 0.57%, and 4.47%, respectively. The highest yield was obtained by the macerate extract with methanol 13.11%.

The DPPH radical-scavenging assay is a widely used method to evaluate the ability to scavenge free radicals generated from DPPH reagent.

DPPH, a stable free radical with a purple color, changes into a stable yellow compound on reacting with an antioxidant. The radical scavenging activity of differents extracts of *T. claveryi*, determined by DPPH radicals is shown in Table 1 in comparison with the control (α-tocopherol).

In this study, the antibacterial and antifungal activities were tested with the dichloromethane, chloroform, ethyl acetate and methanol extracts of *T. claveryi*. The results of the agar well diffusion method of *T. claveryi* extracts using different polarity solvents were shown in Table 2. Among the different extracts, the chloroform extract of *T. claveryi* showed significantly high antimicrobial activity against both Gram-positive and Gram-negative bacteria than the other extracts ($p<0.05$). The largest zone of inhibition (31.66±2.51 mm) was found to be exhibited against *E. coli*. For this microorganism the minimum bactericidal concentration (MBC) was 12.5 mg/mL as shown in Table3. Only the chloroform extract was active against *Corynebacterium* (16±1) and it had MIC of 25 mg/mL.

Table 1 Scavenging effect on DPPH free radical of *Terfezia claveryi*.

Sample concentrations (mg/mL)	DPPH radical scavenging (%)					
	Dichloromethane	Chloroform	Ethyl acetate	Methanol	Macerate	α Tocophérol
40	29.38±0.72[a]	Negatif	Negatif	90.48±0.56[a]	71.36±0.39[a]	91.26±0.14
25	9.12±0.21[b]	Negatif	Negatif	79.21±1.42[b]	50.69±0.56[b]	90.10±0.04
15	5.30±1.23[c]	Negatif	Negatif	71.62±0.88[c]	31.08±0.40[c]	89.28±0.12
5	1.54±0.72[d]	Negatif	Negatif	32.29±0.92[d]	17.74±0.60[d]	88.80±0.11
IC50	ND	ND	ND	8.56 mg/mL	22.16 mg/mL	ND

Values are the mean of three replicates (mean ± SD). Mean values followed by different small letters indicate significant differences (Duncan's test, $p < 0.05$), between different concentrations, for each solvent. ND Not determined.

Table 2 Screening of antimicrobial activity of *Terfezia claveryi* in comparison with standard antibiotic (mean ± SD).

Tested micro-organisms	Inhibition zones diameter (mm)					
Gram-positive bacteria	Dichloromethane	Chloroform	Ethyl acetate	Methanol	Macerate	Streptomycin 30 µg/disc
S. aureus	13±1[ad]	18.33±2.08[a]	10.33±0.57[a]	11±1[a]	06±0[a]	40.66±0.57
B. subtilis	12±1[ad]	25±4[b]	15±1[b]	17±2.64[b]	06±0[a]	40±2.82
E. feacalis	16±1[b]	16±2.64[ae]	14.66±0.57[b]	10±1[a]	06±0[a]	27.5±2.88
Corynebacterium	6.66±1.15[c]	16±1[ae]	06±0[c]	06±0[c]	06±0[a]	30.5±1
Gram-negative bacteria						
P. aeruginosa	18±2[b]	15±2[ae]	14.33±0.57[b]	6.66±1.15[c]	06±0[a]	38.75±1.5
E. coli	17.33±2.51[b]	31.66±2.51[c]	13.66±0.57[b]	13.66±1.52[d]	06±0[a]	37±2.64
P. vulgaris	06±0[c]	06±0[d]	06±0[c]	06±0[c]	06±0[a]	31.66±2.88
S. typhi	14±1[a]	14±1[ae]	23.66±3.21[d]	14±1[d]	06±0[a]	33±1.75
K. pneumoniae	13±1[ad]	12.33±0.57[e]	06±0[c]	06±0[c]	06±0[a]	23.75±3.50
Fungi						
C. albicans	11±1[d]	14.33±0.57[ae]	18.66±1.52[e]	13.66±1.52[d]	06±0[a]	38.25±1.70

Values, including diameter of the filter paper disc (6.0 mm), are means of three replicates.
Mean values followed by different small letters indicate significant differences (Duncan's test, $p < 0.05$), between different concentrations, for each solvent.

The MIC and MBC values of the strains tested were in the range of 12.5-100 mg/mL and 25-200 mg/mL, respectively. These results are presented in Table 3.

Discussion

The increase in the antiradical activity against the DPPH depends significantly on the concentrations, generally we observed that the DPPH radical-scavenging effect increased as the concentration of the extract increased. This activity can be assessed by determining the IC_{50} (Inhibitory Concentration 50) which is a measure of the effectiveness of a given compound to inhibit a biological function or biochemical specific. We have found that the methanolic extract of *T. claveryi* showed significantly the highest antioxidant activity (90.48%) as stated by Hamza et al. [15] at a concentration of 40 mg/mL and the IC_{50} value was 8.56 mg/mL, followed by the macerate extract (71.36%) and the IC_{50} was 22.16 mg/mL. The dichloromethane extract showed the lowest activity (29.38%) while both chloroform and ethyl acetate extracts showed no antioxidant activity to the concentrations tested.

The chloroform and ethyl acetate extracts showed appreciable antibacterial activity against bacteria and yeast. The methanolic extract had moderate antibacterial and antifungal activity but the macerate extract was inactive against all the

Table 3 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Terfezia claveryi* (mg/mL).

Tested micro-organisms	Dichloromethane		Chloroform		Ethyl acetate		Methanol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria								
S. aureus	100	200	25	50	200	200	50	100
B. subtilis	100	200	25	50	50	100	50	100
E. feacalis	50	100	25	50	50	100	50	100
Corynebacterium	ND	ND	25	50	ND	ND	ND	ND
Gram-negative bacteria								
P. aeruginosa	100	200	25	50	50	100	ND	ND
E. coli	100	200	12.5	25	50	100	50	100
P. vulgaris	ND	ND	ND	ND	ND	ND	ND	ND
S. typhi	50	100	12.5	25	25	50	50	100
K. pneumoniae	50	100	50	100	ND	ND	ND	ND
Fungi								
C. albicans	100	200	25	50	50	100	50	100

ND : not determined because the sample was not active by the agar well diffusion test.

strains tested whereas *P. vulgaris* showed resistance to all the extracts of *T. claveryi*.

In the present study, the MIC values of different extracts of *T.claveryi* were lower than the MBC values suggesting that, the *T. claveryi* extracts were bacteriostatic at lower concentrations but bactericidal at higher concentrations (Table 3).

Furthermore, we notice that the antimicrobial activity of *T. claveryi* is greatly influenced by the method of extraction (Maceration and soxhlet extraction). Also we observed that the nature of solvents used has significantly an impact on the antimicrobial and antioxidant activities.

Conclusion

In the present work, it had been concluded that the extraction method and different solvents used had a significant impact on the antimicrobial and antioxidant activities of *T. claveryi*. In terms of extraction method applied, soxhlet extration was the more effective technique compared to maceration extration. Moreover, it was noted that different extracts of *T. claveryi* showed significant free radical scavenging activity and marked antimicrobial activities revealing their potentials for therapeutic uses. However, the identification, characterization and purification of their bioactives compounds will be the aim of further investigation.

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