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Assessment of antimicrobial activity and mycelium insights of *Terfezia claveryi* truffle harvested from Tindouf desert

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ABSTRACT

This work focuses on the bioactivity characterization of the edible desert truffle *Terfezia claveryi* harvested from the Tindouf area in the South–West of Algeria. We investigated the morphological and germination features of *T. claveryi* spores under light and scanning electron microscopy. Afterward, we isolated the mycelium from a unique spore, which was then used in seedling-optimized *in vitro* biomass liquid culture. After that, we focused on the antibacterial and antifungal properties of this mushroom (fruit body flesh and mycelium) evaluated respectively by disc Agar diffusion method and Denis and Webster methods I and II. Furthermore, we tested antagonism against plant-pathogenic fungal species using three confrontation methods on a solid medium. Finally, we developed undescribed protocols for extracting, purifying, and describing bioactive compounds. Results showed that developing *T. claveryi* spore germination, mycelium isolation, and axenic liquid subcultures is very slow. The flesh extract is only antibacterial, while the one from the culture medium is antifungal and antibacterial. The most bioactive extract on bacteria, yeast, and plant-pathogenic fungal strains tested is the one from the mycelium of *T. claveryi*. The latter strain showed good antagonistic power against some plant-pathogenic fungal tested strains, probably owing to antibiotic component biosynthesis with antifungal ability.

INTRODUCTION

Terfez or Terfass or Kamés ("desert truffles") are seasonal edible hypogeal fungi (Ascomycetes, Pezizales) that have been exclusively harvested in wild areas for hundreds of years [1]. They live in symbiotic mycorrhizal associations [2] with herbaceous or shrubby plants of the *Cistaceae* family, especially of the genus *Helianthemum*. These fungi grow in all arid and semi-arid regions around the Mediterranean (Maghreb, southern Italy, southern Spain, and Portugal) and in the Middle East (Iran, Iraq, Syria, Palestine, Kuwait, and Saudi Arabia) [3–11]. The most common method of harvesting desert truffles is observing the ground, as it is often swollen and cracked at the base of the host plant [9].

In Algeria, it is represented by the three genera: *Terfezia* ("Red Terfez"), *Tirmania* ("White Terfez"), and *Picoa* ("Jouaber"); 11 species are known so far in our country [12,13]. These species are distributed in the steppe and Saharan areas. In these regions, Terfez are appreciated for their edible value and properties in traditional medicine: their effects against eye diseases of bacterial origin have been reported for several centuries by the Arabs of ancient times [14] and by a Muslim religious Hadith. "Truffles are like Manna, and their water heals eye diseases" (PBUH Sahih al-Bukhari 5708). By identifying their bioactive components, such as phenolics, terpenoids, polysaccharides, fatty acids, and ergosterols, researchers have paid attention to their biological activities, including antioxidant, antibacterial, and antifungal activities [15]. The chemical content of Terfez has so far been studied with regard to the dietary elements [16–19], demonstrating that

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this fungus provides an excellent dietary protein intake, it is rich in essential amino acids, various fatty acids and, vitamins [20,21]. On the other hand, to the best of our knowledge, despite repeated attempts at isolation, identification, and evaluation of mycelium-active ingredients responsible for the bioactivity of *Terfezia claveryi* from Algeria, a few works have been done [22–26].

Therefore, to increase the knowledge about the bioactivity potential of *T. claveryi* harvested for the first time from the Tindouf area, the current study aims to present experimental optimization of extraction and fractionation of raw extracts from fruit body flesh, mycelium, and its culture medium, and then, the demonstration of antimicrobial active ingredients by *in vitro* tests on several plant-pathogenic fungi and bacteria through mycelium isolation from ascospores of *T. claveryi,* highlighting the antibacterial and antifungal activities of mycelium extracts, culture filtrate, and flesh; investigating the *in vitro* antagonism phenomenon between Terfez and plant-pathogenic fungi; and finally we present organic extracts fractionating process using various chromatographic methods, to achieve active components isolation and chemical characterization.

MATERIALS AND METHODS

Chemicals and instruments

Organic solvents were purchased from *SDS* (F-Asnières sur Seine) and distilled before use. For thin layer chromatography (TLC), we used a chromatographic tank and Ultra Violet (UV) cabinet with a Camag UV lamp De Luxe 29000 (254 and 366 nm) was used. The thin-layer system was silica gel with UV indicator (Si 60 F 254, Merk). The high-performance liquid chromatography (HPLC) apparatus included a Constrametric III (LDC/Milton Roy) pump, a Rheodyne Inc. injector, and Spectro-Monitor D (LDC) UV modules.

Samples collection

Desert truffle fruiting bodies were collected from the region of Tindouf city situated in the Western South desert of Algeria (27′40′33.964″ N 8°7′39.549″ W) (Fig. 1) during the spring season (February–April). As soon as they arrived at the laboratory after 24 transportation hours, samples were gently cleaned to take off sand particles, and their surface was rapidly flamed with ethyl alcohol for disinfection. Truffle samples were separated into two batches, the first to be tested directly as "fresh gleba" and the second for storage for 2 months at room temperature in a dry and dark place, disposed separately on absorbent paper to avoid wilting, to be explored as "dried gleba" samples.

T. claveryi identification

After macroscopic observation of the fruit bodies' aspect, light microscopy (LM) and scan electron microscopy (SEM) were performed to identify ascomycetes and spores' characteristic features. A small amount of desiccated *Terfezia* ascocarp fragment was deposited in a drop of water between the slide and cover slip. For SEM, powder from grounded desiccated ascocarp section was fixed on double-sided tape,

gold coated, and kept under vacuum overnight, then observed in a SEM GEOL JSM 6300 at Nantes University, Faculty of Medicine, Sciences and techniques, Odontology, CHU, Service Commun de Microscopie Electronique, Microcaractérisation et Morpho-histologie-imagerie fonctionnelle (SC3M).

Monoclonal mycelium isolation of *T. claveryi*

Mycelia were isolated, according to Chevalier [27]. The method consisted of taking fragments of gleba from previously alcohol-disinfected dried ascocarps. The fragments were rehydrated in sterile distilled water and ground to obtain a suspension of ascospores. This was later stirred using a vortex. Three to four drops of the suspension were spread on the surface of 1% Malt extract agar poured into Petri dishes, which were firmly sealed with adhesive tape and incubated at 25°C. The ascospores were regularly observed under LM to follow germination stages. Germinated spores were localized under LM and delicately collected to be transferred separately in fresh Malt extract Agar medium Petri dishes to obtain monoclonal strains. Incubation was carried out at 25°C.

Flasks containing 150 ml of 1% Malt extract liquid medium were seeded with a 15 mm square implant collected from *T. claveryi* monoclonal pre-cultures aged 60 days. Once sealed, flasks were incubated on a rotary stirrer (50 rpm) at 25°C.

Bioactivity exploration

To be compared, bioactive metabolites of *T. claveryi* have been prospected in different extracts obtained with other methods. "Soxhlet gleba" extract, "Culture broth" extract, and "Mycelium" extract are symbolized respectively hereafter "*Soxhlet, Culture medium, Mycelium (SCM)*" extracts for text lecture simplification.

Fresh gleba Soxhlet extract (S extract)

A mass of 15 g of fresh gleba taken from ascocarps of *T. claveryi* was cut into small fragments and put in a Soxhlet cartridge. The Soxhlet flask, containing 250 ml of EtOAc, was heated at 50°C for 3 hours. The solvent was removed under vacuum using a Rotavapor at 50°C. The remaining crude extract was dissolved in 2 ml of EtOAc and weighed after complete solvent evaporation.

Culture broth extract (C extract)

After 8 weeks of incubation, filtration through a sintered glass funnel containing filter paper separated mycelial monoclonal culture in a liquid medium from the culture broth. The mycelial mass was drained and weighed. The broth was collected directly into a flask and extracted with EtOAc. One liter of culture filtrate and 150 ml of EtOAc were introduced into a separating funnel, and the mixture was slowly stirred (the funnel valve must be open to escape gases). After decantation, the organic phase was collected in anhydrous sodium sulfate (Na_2SO_4). The aqueous phase was extracted two more times with 150 ml of EtOAc. The three organic phase fractions were mixed, and EtOAc was removed using a Rotavapor at 50°C. The remaining crude extract was dissolved in 2 ml of EtOAc and weighed after the solvent's total evaporation.
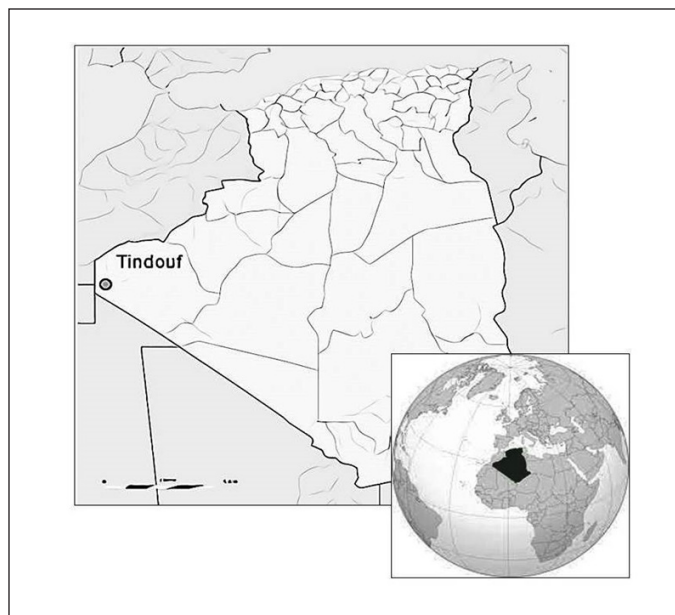
Figure 1. Geographical position of Tindouf city (Algeria).

Mycelium extract (M extract)

The recovered mycelium was put in EtOAc and ground for 5 minutes in an electric mixer, then filtered through cotton and filter paper. The final filtrate was collected in a flask containing a few grams of Na_2SO_4. After Rotavapor concentration at 50°C, the brownish residue was taken up in a few milliliters of EtOAc and weighed after solvent total evaporation.

Antibacterial activity

Nine bacterial strains were tested: *Escherichia coli* ATCC 25922*; Pseudomonas aeruginosa* ATCC 27853*; Streptococcus* ATCC 29212*; Staphylococcus aureus* ATCC 25923*; S. aureus* Rubber Oxygenases A (ROXA) 97009108*; Pseudomonas fluorescens; Salmonella typhi; Klebsiella* sp. and *Shigella* sp. This was performed using the disc Agar diffusion method. One milliliter of standardized bacterial suspensions at 10^6 CFU/ml was spread out over the surface of Mueller-Hinton Agar 4 mm depth poured in Petri plates. The latter was left close to a Bunsen burner for about 20 mn to dry the medium surface. Sterilized discs of 6 or 10 mm made of Whatman n°1 paper were impregnated separately with SCM extracts. They were aseptically applied to the surface of Agar plates at well-spaced intervals. Control discs were impregnated with a sterile 1% Malt extract medium. The plates were incubated at 37°C for 16 hours.

Border-bioactivity assessment of *T. claveryi* EtOAc mycelium extract

Serial dilutions of bioactive EtOAc crude were made to obtain decreasing concentrations as follows:

Crude concentration (mg/ml)	20	10	5	2.5
Concentration/disc (mg/disc)	0.8	0.4	0.2	0.1.

The inhibitory effect of each concentration was tested on five bacterial ATCC and ROXA species (Section "Antibacterial activity") by disc diffusion method. A sterile paper disc was impregnated with 40 µl of one of the dilutions, dried, and then put on the surface of Mueller Hinton agar medium previously inoculated with 1 ml of standardized bacterial suspension (10^6 CFU) in Petri dishes. Incubation was achieved for 16 hours at 37°C.

Antifungal activity tests

Six fungal plant-pathogen strains were tested: *Sclerotinia sclerotiorum*, *Phytophtora* sp., *Aschochyta rabiei*, *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *aalbedinis*, *F. oxysporum* f. sp. *lentii* and one yeast human pathogen strain *Candida albicans*. The testing method used for *C. albicans* was previously described for bacterial tests (Section "Antibacterial activity"), replacing the Mueller-Hinton medium with Sabouraud agar medium [28]. Incubation lasts 24 and 48 hours at 25°C [29].

Dennis and Webster method I

Consisted of digging an 8 mm well in the center of 1% Malt Agar medium in Petri dishes, then filling it with 0.5 ml of SCM extracts. Afterward, the well was covered with an 8 mm fresh active growing mycelium implant of one of the fungal plant-pathogen strains to be tested. Controls were made by filing wells with 0.5 ml of 1% sterile Malt medium or EtOAc before placing the fungal plant-pathogen implant. Petri plates were incubated at 25°C for 7 days.

Dennis and Webster method II

A droplet of 500 µl of one of the SCM extracts was deposited on the surface of the 1% Malt Agar medium poured into Petri dishes. After solvent evaporation, a circular implant of the plant pathogen fungus to be tested (8 mm φ) was deposited 1 cm from the concerned extract. For controls, extracts were replaced by 1% sterile Malt medium or EtOAc. Incubation was at 25°C.

Culture broth mixture test

A mycelial *T. claveryi* culture broth aged at 8 weeks was sterilized by microfiltration on a sterile 0.45 µm Millipore membrane and then incorporated (volume/volume) into Malt (1%) Agar (4%) medium, kept supercooled at 40°C. After homogenization, the mixture was poured into Petri dishes. Succeeding Agar solidification, Petri dishes were inoculated with a circular implant cut from young cultures of one of the six plant-pathogen fungal species tested and incubated at 25°C.

Antagonism of *T. claveryi* mycelium against plant-pathogen fungi

Confrontation on solid medium

Terfezia claveryi mycelium was confronted with *F.o.l., F.o.a., F.o.le*., *Phytophthora* sp., *A. rabiei,* and *S. sclerotiorum*. An 8 mm φ implant of *T. claveryi* mycelium was put on the surface of 1% Malt extract Agar medium and incubated at 25°C. One month later, an 8 mm plant-pathogen fungal strain implant to be tested was placed 3 cm away from the mycelial implant of *T. claveryi*. The control plates were inoculated with

just an implant of the fungal plant-pathogen fungal species to be tested. Dishes were sealed with tape and incubated at 25°C.

Kope and Fortin confrontation method

Malt extract Agar medium poured into Petri dishes was covered with a sterile Millipore membrane (0.45 μm, 5 cm in diameter). An 8 mm φ *T. claveryi* mycelium implant was placed on the membrane. Dishes were sealed with tape and incubated at 25°C. After 3–4 weeks of incubation, the Millipore membrane invaded by Terfez mycelium was aseptically removed and replaced by an implant of one of the plant-pathogen fungal strains tested. For controls, Malt Agar medium dishes were directly inoculated with An 8 mm φ implant of the plant-pathogen fungal species. Petri dishes were incubated for a week at 25°C.

Double layer confrontation method

A mycelial 8 mm implant of *T. claveryi* was placed on 1% Malt extract Agar medium in Petri dishes, which were sealed with tape and incubated at 25°C. One month later, the mycelium was covered with a sterile Millipore membrane (0.45 μm, 5 cm φ), and then 15 ml of supercooled Malt extract Agar medium at 40°C was poured on it. An 8 mm implant of a plant-pathogen fungal species was then placed on the freshly poured medium layer after its solidification. Concerning controls, no Terfez mycelial implant was used first. The sterile Millipore membrane was put on a Malt extract Agar medium layer just before adding the second one, on which the corresponding plant pathogen fungal strain 8 mm implant was placed. Dishes were sealed with tape and incubated at 25°C for a week.

Measurement methods

Antibacterial tests

Bacterial sensitivity to the culture filtrates or to the various Terfez extracts (flesh, culture broth, or mycelium) was appreciated by inhibition zone formation. The inhibition zone diameter around the disk was measured after 24 hours of incubation.

Antifungal tests

Yeast strain *C. albicans* sensitivity was evaluated by measuring the inhibition zone diameter after 24 hours. Plant-pathogen fungal strains: for the tests carried out with Dennis and Webster [30] method I, the mixing broth culture to Malt extract agar medium method, Kope and Fortin [31] method, Millipore membrane double layer method, and the antagonism tests (direct confrontation), the growth of the plant-pathogen fungus was evaluated daily by measuring cultures diameters around the circular implant in two perpendicular directions. Concerning the confrontation tests, the fungal species growth was monitored daily and stopped as soon as the two mycelial fronts came into contact.

For tests performed with SCM extracts of *T. claveryi* by method II of Dennis and Webster [30], the inhibition was evaluated by mycelial growth stopping of plant-pathogen fungus. The inhibition zone diameter was measured in two perpendicular directions. In both cases, the results represent the average of three repetitions, and the percentage of inhibition was calculated according to the following formula [32]: $I = (Dt - Dm)/Dt.100$ where I: % mycelial growth inhibition; Dt: mycelial culture control mean diameter; Dm: mycelial culture mean diameter in the presence of Terfez. Measurements ended when mycelial control cultures covered the entire surface of the medium.

Chemical bioactive compounds characterization

Throughout the fractionation optimization process, antibiogram tests using *P. aeruginosa* ATCC 27853 were performed on all issued fractions to track the bioactive molecules' progression. The following methods were successively used: TLC, liquid/liquid polarity partition, open column chromatography on silica or Sephadex LH-20 column, HPLC, and finally gas chromatography-mass spectrometry (GC-MS). Analytic TLC mode allowed the first extract exploration step. Otherwise, it was used parallel to other techniques to appreciate their efficiency. All obtained fractions were inspected for their chemical composition (TLC) and their biological activity (antibiogram on ATCC bacteria).

TLC on silica gel

Extraction migration was performed in the CH_2Cl_2/MeOH (90:10) mixture and revealed by UV.

Liquid/liquid polarity partition (Kupchan method)

A solution of the initial mixture (Terfez extract) was extracted using a separating funnel. 5 mg of mycelium crude was dissolved in 20 ml of MeOH: H_2O (90:10), and 20 ml of hexane was added. The mixture was carefully stirred and allowed to settle until the appearance of two phases. The methanol phase was put aside, and the hexane phase was extracted twice again as precedent to obtain "Hexane extract." The methanol phase was adjusted with water to 70:30 MeOH: H_2O and extracted 3 times using 20 ml of CH_2Cl_2, which gives the "CH_2Cl_2 extract." Extracts were dried over Na_2SO_4, filtered, evaporated, and weighed.

Open chromatography column

Silica chromatography "step gradient mode"

We used a 1.5 cm φ column filled with 6 g of silica gel to a height of 10 cm (Kiesel gel 60 types, 40–63 μm). 5 mg of extract was deposited at the top of the column. The elution takes place in successive stages with the following solvents (20 ml for each mixture) as follows:

CH_2Cl_2	100	80	50	0%
MeOH	0	80	50	100%.

Obtained fractions were dried over Na_2SO_4, filtered, evaporated, and weighed.

Chromatography on silica "flash" mode

We used the same conditions as above but with a solvent mixture volume of 50 ml and applied a vacuum at the column outlet to accelerate the flow elution rate. Fractions were dried over Na_2SO_4, filtered, evaporated, and weighed.

Sephadex LH-20 chromatography (Pharmacia)

This open-column chromatography requires special conditions without a vacuum. A 2.5 cm φ column was filled to a height of 10 cm with 7.5 g of LH-20 gel previously swollen in the first elution solvent for 2 hours. It must always be soaked in solvent once introduced into the column to prevent it from cracking in the column, which would disturb the fractionation process. 50 mg of crude extract were delicately deposited at the head of the column. The solvent was slowly added. The elution was performed with 60 ml of each of the following solvents as follows:

CH_2Cl_2	100	99	98	95	90	80	50%
MeOH	0	01	02	05	10	20	50%.

Fractions were dried over Na_2SO_4, filtered, evaporated, and weighed.

High-performance liquid chromatography

Sephadex LH-20 issued fractions were analyzed under the following conditions: Diol columns (4,6 × 300 mm Interchim, France) in normal phase, solvent CH_2Cl_2-MeOH 95:5, then 97:3, flow rate 1 ml/mn, injection of 2.5 mg in 500 µl of solvent (5 mg/ml), UV detection at 230 nm.

Gas chromatography-mass spectrometry analysis

GC-MS was carried out on an Agilent 6890 gas chromatograph equipped with a splitless capillary inlet system and an Optima 5 MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) (Macherey-Nagel, Düren, Germany). The carrier gas was helium at an inlet pressure of 2.48 psi. A Hewlett-Packard 7673A liquid autosampler, operated in the fast mode for splitless injection, was used in conjunction with the gas chromatograph. The volume of sample injections was 2 µl. A Hewlett-Packard 5973 mass spectrometer, used as a detector, was operated in electron impact mode at 70 eV in the full-scan mode and directly interfaced with the chromatograph by the capillary column. The oven temperature was started at 50°C (held for 3.0 minutes), followed by a 3°C/minute ramp to 130°C and a second 10°C/minute ramp to 240°C. The injector and transfer line temperatures were maintained at 250°C.

RESULTS

T. claveryi identification

The hypogeous fruiting body of *T. claveryi,* also called ascocarp, have a rounded mass shape, fleshy like a tubercle enveloped by a peridium of reddish-brown color, thin, continuous with a smooth surface with several slits (Fig. 2a). The homogeneous, firm flesh, also called gleba (or glebe), is white in color, tinged with yellow.

As reported by Janex-Favre *et al.* [33], asci contain 3–8 ascospores club-shaped (Fig. 2b). The ascospores of *T. claveryi* measure 16–17 µm in diameter (Fig. 2c); they are spherical, hyaline, and provided with ornamentation in the form of simple nipples with loosely organized contents (Fig. 2d) as described by Malençon [34].
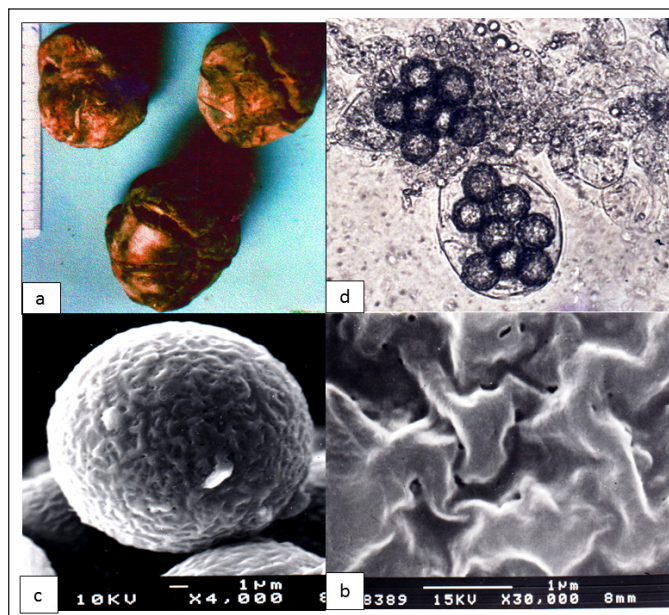
Figure 2. (a) Dark-colored *T. claveryi* ascocarps with small shallow slits. (b) *T. claveryi* asci with eight ornamented ascospores (×1,600). (c) Cross-linked ascospore of *T. claveryi* observed with SEM (×4,000). (d) Details of alveolar ornamentations of *T. claveryi* ascospore (×30,000).

Monoclonal mycelium isolation of *T. claveryi*

The mycelium culture of the hypogeous Ascomycete *Terfezia* is hard to obtain [35]. Spore germination of *T claveryi* ascospores, as for other species of the same genus, is preceded by a latency phase of variable duration (3 months in our study).

Initially, each ascospore releases a large, highly refractive lipid globule (Fig. 3a). We observed the extrusion of one or two germ tubes that emerge from any point of the ascospore (Fig. 3b), which elongate and ramify after that (Fig. 3c). The hyphae of *T. claveryi* are septate and have many branches (Fig. 3d and e).

The mycelium is characterized by slow rate growth and is intra-matrix organized on Malt extract Agar medium. After 6 weeks of culture, its color varies from yellow to brown (Fig. 4a). In a shacked liquid culture medium, the mycelium makes a submerged compact and dense mass of brown color with a shallow supernatant face of the same feature as that grown on a solid medium (Fig. 4b).

Bioactivity exploration of different *T. claveryi* extracts

Fresh gleba Soxhlet extract (S extract)

Fresh EtOAc gleba S extracts were tested on three bacterial strains (*P. fluorescens, S. aureus,* and *S. typhi*). *P. fluorescents were* insensitive, while *S. typhi* and *S. aureus* were slightly sensitive (Fig. 5a and b). The inhibition zone diameters were 15 and 17 mm, respectively. *Candida albicans* was also faintly sensitive to this extract (Fig. 5c).

Culture broth extract (C extract)

The crude extract is brown, turning to very dark pink. The four bacterial tested strains, *P. fluorescens*, *S. aureus, E.*
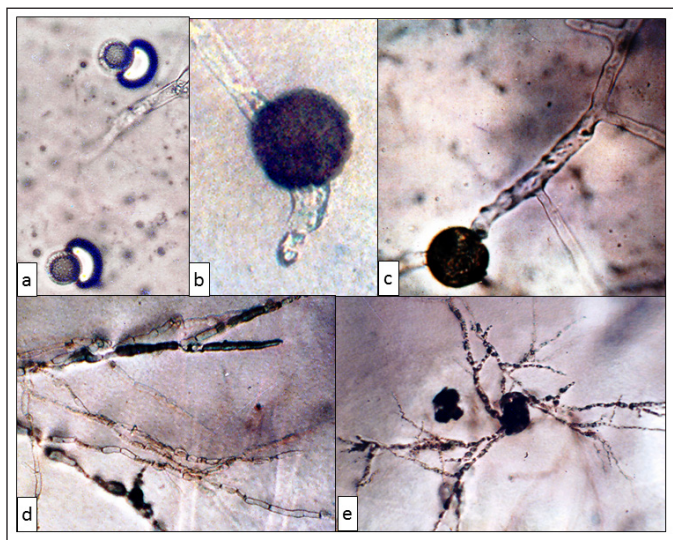
Figure 3. (a) Ascospores of *T. claveryi* in early germination stages, showing the release of refractive lipid globules (×1,000). (b) Ascospore germination with two germinative tubes (×750). (c)–(e) First *T. claveryi* hyphae branching (×1,000), (×100), and (×10).

coli, and *S. typhi,* were sensitive to this extract (Fig. 6a–c). The inhibition diameter zones ranged from 20 to 29 mm (with 10 mm φ paper test discs). *Terfezia claveryi* culture filtrate extract affects the growth of *C. albicans*. The inhibition zone diameter was 23 mm (with test discs of 10 mm *φ*) (Fig. 6d).

For the plant-pathogen fungal strains bioactivity tested with Dennis and Webster method I, three species tested by the well method*, F.o.l., F.o.a,* and *F.o.le,* were sensitive to the culture filtrate extract with respective inhibition percentages of 22.3%, 21.7%, and 24.3%. *Phytophthora* sp. was strongly inhibited by this extract, with a percentage of inhibition exceeding 89%. The other fungal species tested (*S. sclerotiorum* and *A. rabiei*) did not show sensitivity to this extract. Tested with method II of Dennis and Webster, only three fungal species (*F.o.a., F.o.l.,* and *F.o.le.*) are sensitive to the extract of the culture filtrate deposited near the implant; the inhibition percentages were 14.6%, 15%, and 16.3%, respectively. At the same time, the greatest inhibition was observed for *Phytophthora* sp. with a percentage inhibition of 48.3%.
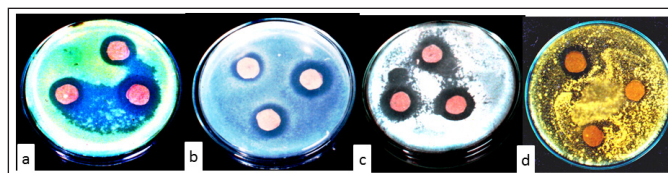
Mycelium extract (M extract)

EtOAc extract had an inhibitory effect on the growth of all the bacterial species tested: *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *S. aureus* ROXA 27009108, *E. coli* ATCC 25922, *Streptococcus* ATCC 29212, and *S. typhi* (Fig. 7a–e). Its activity on bacteria was maintained even after a freezing period of more than 20 days. *Candida albicans* was sensitive to this extract, too, with an 18 mm inhibition zone average diameter. Concerning its effect on plant pathogen fungal species, with method I of Dennis and Webster, the growth of the three special forms of *F. oxysporum* was slowed down in contact with the mycelial extract of *T. claveryi*. The inhibition percentage was 23% for *F.o.l*, 22.2% for *F.o.le,* and 25% for *F.o.a. Phytophtora* sp. was also susceptible to this extract; its growth was inhibited by 86.6%. The remaining fungal strains tested had an average growth rate. But using method II of Dennis
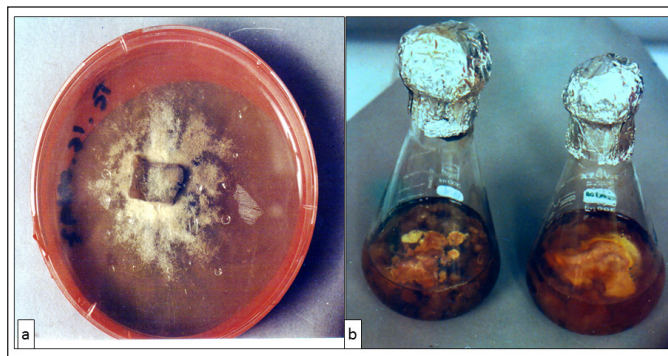
Figure 4. (a) Macroscopic aspect of *T. claveryi* mycelium grown on Malt extract agar-based medium. (b) Static (left) and agitated (right) cultures *of T. claveryi* in liquid Malt extract-based medium.
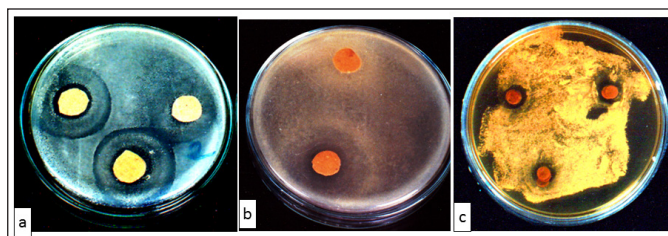
Figure 5. (a) Growth inhibition of *S. typhi* in the presence of *T. claveryi* flesh Soxhlet extract. (b) Inhibition of *S. aureus* growth in the presence of *T. claveryi* flesh Soxhlet extract. (c) Inhibition of *C. albicans* growth in the presence of *T. claveryi* flesh Soxhlet extract.


Figure 6. (a) Growth inhibition of *P. fluorescens* in the presence of *T. claveryi* EtOAc culture medium extract. (b) Growth inhibition of *S. aureus* in the presence of *T. claveryi* EtOAc culture medium extract. (c) Growth inhibition of *S. typhi* in the presence of *T. claveryi* EtOAc culture medium extract. (d) Growth inhibition of *C. albicans* in the presence of *T. claveryi* EtOAc culture medium extract.

and Webster, the mycelium extract had a weak inhibitory effect on the growth of *F.o.l.*, *F.o.le.,* and *F.o.a.*, where percentage inhibitions of 6.3%, 6.13%, and 7% were recorded, respectively. Its effect was nil on the growth of *S. sclerotiorum* and *A. rabiei*. The strongest growth inhibition effect was observed on *Phytophthora sp*. with a percentage of 39%.

Culture broth mixture test

Concerning the method of mixing *T. claveryi* culture broth to 1% Malt extract Agar medium, among the six fungal species tested, only *F.o.l., F.o.le.,* and *F.o.a.* showed growth slowdown compared to control cultures. The inhibition percentages for each species were 18.5%, 19%, and 20.7%, respectively.
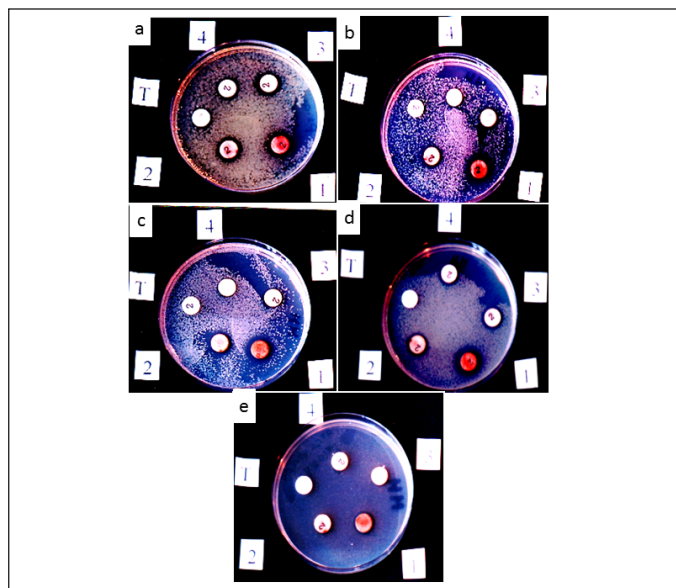
Figure 7. (a) *Pseudomonas aeruginosa* American Type Culture Collection (ATTC) 27853 growth inhibition and bioactivity borderline of EtOAc *T. claveryi* mycelium extract: 1 = 20 mg/ml; 2 = 10; 3 = 05; 4 = 02.5, (b) *S. aureus* ATCC 25923 growth inhibition and bioactivity borderline of EtOAc *T. claveryi* mycelium extract: 1 = 20 mg/ml; 2 = 10; 3 = 05; 4 = 02.5, (c) *S. aureus* ROXA97009108 growth inhibition and bioactivity borderline of EtOAc *T. claveryi* mycelium extract: 1 = 20 mg/ml; 2 = 10; 3 = 05; 4 = 02.5, (d) *E. coli* ATCC 25923 growth inhibition and bioactivity borderline of EtOAc *T. claveryi* mycelium extract: 1 = 20 mg/ml; 2 = 10; 3 = 05; 4 = 02.5, and (e) *Streptococcus* ATCC 29212 growth inhibition and bioactivity borderline of EtOAc *T. claveryi* mycelium extract: 1 = 20 mg/ml; 2 = 10; 3 = 05; 4 = 02.5. T = controls.

Border bioactivity assessment of *T. claveryi* EtOAc extract

The five tested bacterial strains were sensitive to mycelium organic extract. *Pseudomonas aeruginosa* ATCC27853 and *E. coli* ATCC 25922 were inhibited by concentrations below 2.5 mg/ml (Fig. 7a and d). *Streptococcus* ATCC 29212, a very resistant strain to many antibiotics, was the less sensitive, with a limited dose of 10 mg/ml (Fig. 7e). *Staphylococcus aureus* ATCC 25923 and *S. aureus* ROXA 97009108 had a moderate sensitivity compared to the other bacterial tested strains (Fig. 7b and c).

Antagonism of *T. claveryi* mycelium against plant-pathogen fungi

Confrontation on solid medium method

After 15 days of incubation, the growth of *F.o.a., F.o.l.* (Fig. 8), *F.o.le.* and *Phytophthora* sp. confronted with *T. claveryi* were completely inhibited throughout the experimentation period. The percentage of inhibition was 100% for the four plant-pathogen fungal species. However, *S. sclerotiorum* and *A. rabiei* had an average growth rate, and the percentage of inhibition was trivial.

Kope and Fortin confrontation method

The growth of *F.o.l* (Fig. 9)*, F.o.a., F.o.le.,* and *Phytophthora* sp. was totally inhibited by diffusible metabolites
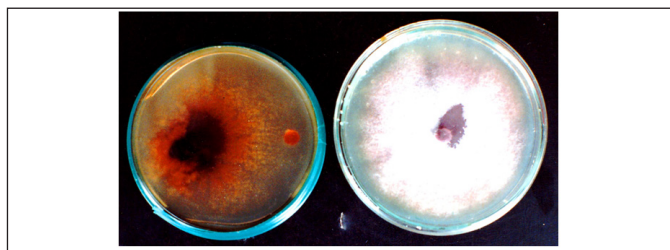
Figure 8. Confrontation of *T. claveryi* mycelium with *F. oxysporum* f.sp. *lycopersici* showing total growth inhibition of the fungal plant-pathogen strain. Dish on the right = control.
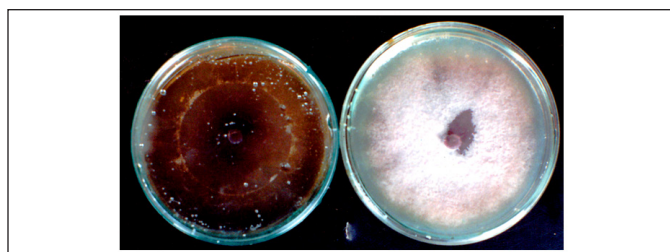
Figure 9. Growth Inhibition effect of *T. claveryi* on the growth of *F. oxysporum* f.sp. *lycopersici* tested by [30]. Dish on the right = control.

through the Millipore membrane; the inhibitions percentage was 100%. Those metabolites didn't affect the growth of *A. rabiei* or *S. sclerotiorum*; the inhibition percentage was 0% for these fungal species.

Double layer confrontation method

Phytophthora sp. growth was totally inhibited throughout the incubation period by 100%.

The metabolites of *T. claveryi* were active on the growth of *F.o.l., F.o.a., and F.o.le*, but the percentage of inhibitions were, respectively, 52.9%, 47.2%, and 49%. The other fungal species tested had normal rate growth on this test.

Chemical bioactive compounds characterization

TLC on silica gel

The EtOAc culture broth and mycelium extracts of *T. claveryi* were completely comparable in TLC: two main compound clusters visible under UV (360 nm). Phosphor-molybdenum reagent spraying revealed a red spot followed by a blue one and 6 other brown spots. It is very probable that *T claveryi* mycelium synthesizes and releases some of these products in his proximate environment. These substances were more present in the mycelium than in the culture broth: the broth extract was entirely used for the TLC, while we put aside a significant amount of mycelium extract, allowing further fractionation investigations.

Liquid/liquid polarity partition

We obtained middling results. A weak activity was found in the two extracts, "CH_2Cl_2," and "hexane," with the same inhibitory action on *P. aeruginosa* ATCC 27853 and *E. coli* 25922.

Open chromatography column

Silica chromatography "step gradient mode"

From the first column, three fractions were collected corresponding to metabolite clusters. In the two first fractions, we detected the same metabolite clusters visible under UV in TLC; they were free of bioactivity. The third eluted fraction was invisible under UV and bioactive on *P. aeruginosa* and *S. typhi*. Fractions 2 and 3, collected from the second column, were bioactive on *P. aeruginosa* ATCC 27853.

Chromatography on silica "flash" mode

The first three fractions were bioactive on *P. aeruginosa* ATCC 27853. This column was qualitatively correct, and there were no product losses.

Sephadex LH-20 chromatography

Fractions 1 and especially 3 and 4 were active against *P. aeruginosa* ATCC 27853. The LH-20 allowed us to recover good bioactivity; its resolving power was higher than that of silica. According to TLC analysis and the biological test results, we selected the last two methods, leading to a good bioactivity recovery. However, the LH-20 method leads to a better repartition of the metabolites in the fractions, the raison, because we choose it to improve the fractionation protocol. We noticed at least two different active ingredients or two clusters of active ingredients. Based on the eluting solvent nature, these active principles were relatively polar since they migrate only with high percentages of MeOH in the solvent. The subsequent fractionation steps require a more resolving method: HPLC, which we applied to fractions 3 and 4 from the LH-20 column (these fractions were extracted with 2% and 5% MeOH in CH_2Cl_2).

High-performance liquid chromatography

A first attempt (CH_2Cl_2:MeOH 95:5) showed that this mixture is too eluent. However, with the proportions 97:3, we obtained a suitable peak spread (Fig. 10). The recordings show the presence of major products: two in fraction number 3 (LH-20) and three in fraction number 4. It is early to declare that they correspond to the active ingredients, especially since the highest UV peaks do not necessarily compare to the most abundant products. But we now have a fractionation method that is settled and, therefore, applicable on a larger scale.

At the end of this characterization process, we attempted GC-MS analysis to characterize the chemical nature of the bioactive *T. claveryi* compounds. Unfortunately, we recovered only the HPLC fraction of three prominent peaks in tiny amounts because of successive extract depletion. In addition, HPLC fraction 4 amounts were inoperative.

Gas chromatography-mass spectrometry analysis

The GC spectrum of collected HPLC fraction 3 main's components revealed a major peak at 10.06 minutes (Fig. 11). Its mass spectrum (Fig. 12) corresponds mostly to benzoquinones, according to the Agilent software database.

DISCUSSION

Terfezia claveryi ascospores have very weak germination ability, characterized by a latency phase of unknown
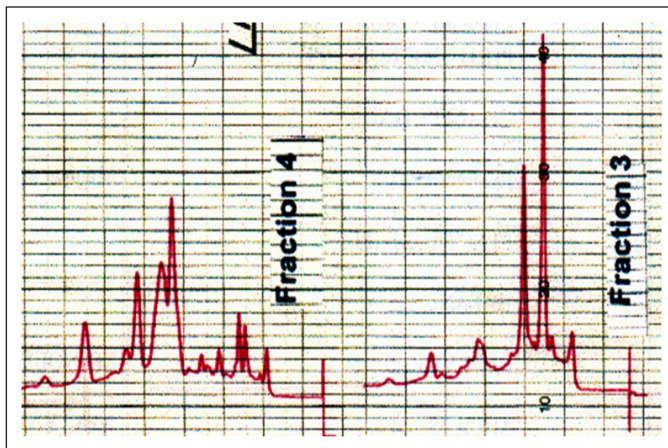

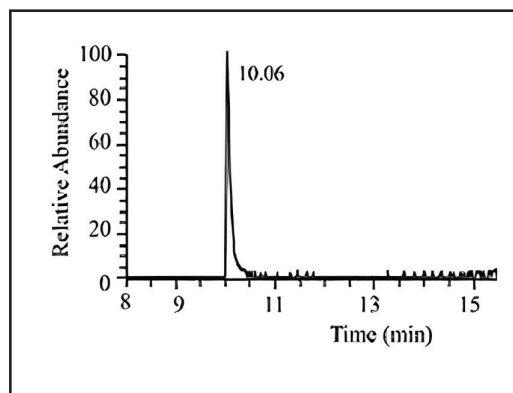**Figure 10.** HPLC spectrum of bioactive fractions 3 and 4.

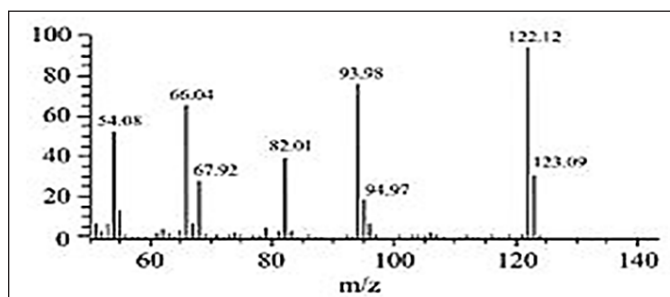**Figure 11.** GC analysis of HPLC issued fraction 3 main's peak.

Figure 12. Mass spectrum of GC peak at 10.06 of HPLC gave fraction 3 main's peak.

nature. This dormancy ascospores phenomenon could be of constitutive origin (spore's wall thickness preventing nutrient penetration) or exogenous (lack of specific growing factors) [36]. Terfez spores' germination factors are not understood [5,37]. The germinative bud of *T. claveryi* emerges from no particular area of the spore, as noted by Awameh and Alsheikh [38] and Fortas and Chevalier [5], unlike the ascospores of the genus *Tuber,* which have a particular germinative pore [39–43]. We also noticed that the *Terfezia* spores' suspension used for the mycelium isolation was free of contaminating germs (bacteria, yeasts, and fungi), comforting the hypothesis that Terfez could

therefore contain antibiotic material. Nevertheless, given the low yields of mycelia obtained using traditional culture methods of this truffle [44], it remains crucial to find an adequate culture medium and to improve the culture conditions to solve this drawback. In this aim, the work carried out by López-Nicolás *et al.* [45] confirmed the ability of cyclodextrins to stimulate the growth of *T. claveryi* mycelium when present in the culture medium, while Navarro-Ródenas *et al.* [46] reported that slight water stress (−0.45 MPa) could improve mycelial inoculum production of two desert truffles strains, *T. claveryi* and *Picoa lefebvrei*.

The three special forms of *F. oxysporum, F.o.l, F.o.le., and F.o.a.,* tested by mixing the culture broth filtrate with the culture medium of *T. claveryi* show growth slowdown compared to controls, hence a low sensitivity to the substances present in the culture broth. The other species were not inhibited. These results differ from those of Dennouni [25], who revealed that the culture broth of two *Tirmania* species (*Tirmania nivea* and *Tirmania pinoyi*) incorporated into the culture medium of plant-pathogen fungal species inhibits their growth. In her study, the percentage inhibition was 50% for *S. sclerotiorum*, but bacteria were weakly sensitive. Dried or fresh gleba extracts obtained by different solvent interactions had no effect on the growth of bacterial and fungal tested species. These results differ from those of Rougieux [22] with *Terfezia boudieri* and Dennouni [25] with *T. nivea* and *T. pinoyi*.

In contact with fresh gleba Soxhlet extract obtained, two bacterial species were slightly sensitive. *Pseudomonas fluorescens*, strongly inhibited by the extract of gleba from *T. pinoyi* and *T. nivea* when studied by Dennouni [25], was insensitive to the extract of fresh gleba of *T. claveryi*. The extract of dried gleba obtained using Soxhlet was inactive on *E. coli, S. typhi, P. fluorescens, P. aeruginosa, Klebsiella* sp., and *Shigella* sp., as well as on *C. albicans*. The *T. claveryi* fresh gleba, therefore, contains low concentrations of active products on tested bacteria and fungi. Similar results were obtained by Chellal and Lukasova [47] and Dennouni [25], who respectively tested the extracts of *T. boudieri* and *T. nivea* on the growth of various bacterial and fungal species. Conversely, the culture filtrate extract with EtOAc strongly inhibits bacterial and yeast-like tested species. Species susceptibility in descending order is scored as follows: *E. coli > S. typhi P. fluorescens > S. aureus*. *Candida albicans* was also inhibited by this extract.

Culture filtrate extract effect on plant-pathogen species growth was variable; *Phytophthora* sp. was strongly inhibited by methods I and II of Dennis and Webster [30]; on the other hand, *F.o.l., F.o.a.,* and *F.o.le*. Showed a lower inhibitions percentage with method I than with method II. These results agreed with a [25] study that revealed that culture filtrate extract of *T. nivea* with EtOAc inhibits the mycelial growth of *Phytophthora* sp. Otherwise, EtOAc extract was active on all the bacterial strains tested and even on *Streptococcus* ATCC 29212, a hospital strain with several antibiotic resistance. The susceptibility of the bacteria tested, in decreasing order, was as follows: *S. typhi > E. coli > P. fluorescens S. aureus > E. coli* ATCC 25922 = *S. aureus* ATCC 25923 > *P. aeruginosa* ATCC 27853 = *S. aureus* ROXA 97009108 > *Streptococcus* ATCC 29212. With method 1 of Dennis and Webster [30], the mycelial extract had

a strong inhibiting effect on *Phytophthora* sp., *F.o.a., F.o.l*. and *F.o.le*. growth. Method II of Dennis and Webster [30] gave similar results but lower inhibition percentages. The remaining plant-pathogen fungi tested show no sensitivity. However, the EtOAc mycelial extract had a variable inhibitory effect, depending on the fungal species tested and the techniques used. Kope and Fortin's [31] confrontation methods result, and those of the double layer technique, showed that *T. claveryi* is antagonistic to most of the plant-pathogen fungal tested species. Regardless of the technique used, mycelial growth of the three special forms of *F. oxysporum* (*F.o.l., F.o.a*., and *F.o.le.*) and *Phytophthora* sp. were inhibited. The growth inhibition of *A. rabiei* and *S. sclerotiorum* is weak and almost missing. These results differ somewhat from those of Dennouni [25], who showed that *T. nivea* has a generally weak antagonistic power on plant-pathogen fungal species, particularly *S. sclerotiorum; Phytophthora* sp., and *Phoma* sp. However, a purified aqueous extract of *T. claveryi* presented a very good bioactivity against *E. coli, S. aureus*, and *Staphylococcus epidermidis* as reported by Casarica *et al.* [48], and that of *Tirmania* sp. had a promising antibacterial activity toward *P. aeruginosa* according to Owaid *et al.* [49].

According to Shaw *et al.* [50], in *in vitro* interactions between ectomycorrhizal fungi and plant-pathogen fungi, the culture conditions, the nature of the nutrient medium, and its pH can either stimulate or inhibit fungal growth. For other authors [51], the inhibitory effect of ectomycorrhizal fungi against plant-pathogen fungi depends on factors combination, such as pH, hosting plant, ectomycorrhizal fungus, and the plant-pathogen fungus concerned. The inhibitory variation effect of Terfez against plant-pathogen fungi seems to depend on culture conditions, studied Terfez species, and tested plant-pathogen fungi. The antimicrobial activities of *T. claveryi* seem *in vitro* best demonstrated in culture broth and mycelium EtOAc extracts. Kope and Fortin [31] and double layer methods were the most antagonism displayed. The several biological tests explored herein show clearly that the mycelium of this fungus synthesizes and secretes antibacterial and antifungal substances in both liquid and solid culture media.

This phenomenon is rare [52], knowing that, in general, antibacterial agents are inactive on fungi because of structure differences, organization, and composition of the prokaryotic and eukaryotic cell walls. Our products are, therefore, able to cross a fungal chitin-cell wall and react on fungal cytoplasmic membranes containing sterols (absent in bacteria) [53]. This finding encourages us to try to identify these products; their presence seems important in the mycelium, the gleba, and at a minor level in the culture broth. Developing a fractionation process allowed us to observe that the active fractions were always colored (yellow to red) and of high polarity (solubility and chromatographic behavior). These characteristics, as well as the fungal origin of this species, oriented us to search in the bibliography if comparable substances had already been described, either in the genus *Terfezia* or, more generally, in the plant and fungal kingdoms. Chellal and Lukasova [47] performed EtOAc extractions from fresh *T. boudieri* and *T. pinoyi* gleba. Their results showed that the gleba of *T. boudieri* contains more antibiotic substances than that of *T. pinoyi*. The

greatest antibiotic effect was obtained with a MeOH extract at 45°C on Gram-positive bacteria only; this extract was inactive on *P. aeruginosa*. Extraction at higher temperatures showed that these antibiotic substances, of a proteic nature, were thermolabile. The Retention Factor (RF) of the most active spot is very close to that of pristinamycin, suggesting that it is a streptogramin (also called synergistin) [24]. In the present study, the mycelial extract of *T. claveryi* was thermostable, and Soxhlet extraction and Rotavapor evaporation raised the temperature above 65°C. EtOAc allows antimicrobial substance extraction. The mycelial extract is active on Gram-positive and Gram-negative bacteria (including two strains of *P. aeruginosa*). This lets us suppose that the antimicrobial substances extracted from the gleba of *T. boudieri* and *T. pinoyi* by Chellal [24] are different from those that we extracted from the mycelium and the gleba of *T. claveryi*. Baute *et al.* [54] found a new antibacterial compound of metabolic origin in the gleba of *Terfezia* sp. This compound is only obtained after enzymatic activation and "activator" plasmolytic treatment (drying, freezing-thawing, grinding, and so on) of the α-D-1,4 glucans contained in the Terfez gleba glycogen and starch, resulting in pyrogenic T compounds that do not pre-exist in natural cells.

It seems logical to suppose that the presence or absence of antimicrobial compounds in Terfez could be inducible by aggression factors (cold and heat thermal shock alternations that characterize Terfez preferred regions climate). These molecules increase the intracellular molarity of the mycelium and could thus protect it against freezing [54]. These substances have no common point with ours that seem original and spontaneously present in *Terfezia* mycelium, culture broth, and fresh gleba. Very similar compounds were found [25] in the liquid culture broth of *T. pinoyi* and *T. nivea*. Moreover, by reviewing the main categories of fungal and plant metabolites, we realize the analogy between our products and those of the Quinone class. Quinones are ubiquitous biological pigments found in a range of living organisms (bacteria, fungi, higher plants, and a few animals) [55]. They are oxygenated aromatic compounds biosynthesized either in the polyacetate or shikinic acid pathway. There are several categories of Quinone compounds, mainly according to ring cycle number and oxidation degree:

- Antitumor tetracyclic Quinones in some *Streptomyces*.
- Tetracyclic Quinones: anthraquinones with a purgative action from *Ramnaceae* (Bourdaine, Cascara, and Rhubarb) and *Caesalpinaceae* (Senna).
- Bicyclic Quinones, or naphthoquinones, with antimicrobials and dyes with well-known properties, are present in *Drosera*, *Jugians* (Walnut), and *Lawsonia* (Henna).
- And finally, monocyclic Quinones or benzoquinones of yellow, orange, or red color are poorly soluble in water, well soluble in organic media of high polarity, and strongly antimicrobial. Only this group is stable and is often found in the plant kingdom.

All these Quinones are biologically very active products, acting mainly in biological oxido-reduction phenomena. Anthra and benzoquinones are especially active antibacterial by enzymatic inhibition (addition reaction with thiol and amine groups), antifungals, and sometimes dewormers [56]. Quinones can also be exfoliating and keratolytic, therefore,

usable in specific skin disorders [57], which was also reported by Haloubi and Denizot [14], who mentioned that maceration water of Terfez cures *alopecia areata*. If the connection between our products and the general class of Quinones is clear, we can try to specify to which category they belong. Tetracyclic Quinones are rare and still undescribed in fungi. Anthraquinones are powerful purgatives, irritating the intestinal mucosa [57]. However, we know that excessive consumption of Terfez causes colic, urinary retention, paralysis, and even cardiac arrest [14]. However, Ahmed *et al.* [58] studying the chemical composition and toxicity of *T. boudieri* ascocarps Chat. from Libya did not find such products. Naphthoquinones are closer to the biological behavior of our products, but their colors show up more in an alkaline medium. Finally, there remain the benzoquinones, which are closest in their characteristics to the active principles of *T. claveryi* and are, in addition, widespread fungal pigments [57]. Because of tiny HPLC-issued fractions amounts, GC-MS analysis results were partial. Nevertheless, we were at least able to closely identify HPLC fraction 3 main peaks as strongly related to the benzoquinones class. Terfez could, therefore, contain a multitude of antimicrobial compounds present in the gleba and/or in the mycelium and vice versa or are only synthesized under certain ecological conditions [59].

CONCLUSION

The spore germination study shows that the ascospores of *T. claveryi* have an exogenous dormancy, likely to be lifted by the presence of chemical factors in the culture medium and an endogenous dormancy, which would be linked to the impermeability of the envelope's spores. The mycelium resulting from ascospores germination grows slowly on liquid or solid media, which constitutes a major obstacle for the cultivation development attempts of this mushroom. The massive production of mycelium in Malt extract liquid medium allows the extraction of antimicrobial substances and the carry out of antibiotic tests. The results show that the extracts of mycelium and culture broth had a broad spectrum of action against bacteria, fungi, and yeasts. *Terfezia claveryi* active principles concentration is higher in the mycelium than in the culture broth or gleba. Gleba extracts were only antibacterial. Antagonism tests show that *T. claveryi* could act as a good antagonist against specific fungal species, particularly *Fusarium* genera. Furthermore, we developed a fractionation protocol for the active compounds synthesized by *T. claveryi* mycelium. The outcomes of the various chromatographic techniques and antimicrobial activity tests of the fractions of each technique enabled us to select the LH-20 method, which gave better product separation. HPLC analysis of the active fractions from the LH-20 column reveals the presence of at least two active products probably belonging to Quinones. Ample active fraction amounts are needed to isolate pure products to elucidate their complete chemical structure and to evaluate their pharmacological properties.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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