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Original Article

Bioactivity and Chemical Profiling of Medicinal Fungi *Inonotus cuticularis* and *Inocutis levis* (Hymenochaetaceae) using Chromatography and Mass Spectrometry

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ABSTRACT

Polypore fungi are among the most preventable mushroom-forming fungi with known therapeutic potential, though only a few species have been securitized for their metabolites. This study examines the biological activity and bioactive compounds of *Inocutis levis* and *Inonotus cuticularis* collected in Iran. We examined the antimicrobial, antioxidant, and cytotoxic properties of n-hexane, acetone, and ethyl acetate extracts. Chemical profiles were assessed by chromatography and mass spectroscopy techniques. The acetonic extracts exhibited the highest antibacterial effect against all tested microbial strains. The IC_{50} values for DPPH and ABTS assays ranged from 144.98 – 469.02 µg/mL and 128.06 – 331.52 µg/mL, respectively. The MTT assays for both fungi indicated low toxicity on normal HDF cells with IC_{50} values ranging from 1447 to 1908 µg/mL. HPLC-DAD analysis showed a high level of gallic acid among other detected phenolic compounds. LC-ESI-MS/MS analysis displayed the presence of various sesquiterpenoids, furans, and styrylpyrone-class compounds. Inotilone, inonotin H and C, phellinulin B and M, cinnamic acid, p-coumaric acid, caffeic acid, phelligridin A and D, hispidin, and gallic acid were found in both species. Daedalin A is reported for the first time from the fungal family Hymenochaetaceae. In addition, several volatile compounds, including alkene hydrocarbons and some fatty acids, such as linoleic acid, were detected in GC-MS analyses. We suggest that *I. levis* and *I. cuticularis* have dual antibacterial and antioxidant properties and diverse metabolites, potentially opening new windows in future natural product-based medicine.

INTRODUCTION

Polypores are an ecologically important group of macrofungi possessing diverse compounds with therapeutic potential. However, despite their rich diversity, their bioactivity and metabolites remain far less studied than in higher plants and bacteria, and only a few species have been securitized in this regard [1-3]. Nevertheless, medicinal polypores have been shown to contain diverse molecules, many of which possess scavenger and antimicrobial properties that protect the human body from pathogens and also inhibit cellular oxidation [4].

Inocutis levis (P. Karst.) Y.C. Dai and *I. cuticularis* (Bull.) P. Karst. are two mushroom-forming fungi from the family Hymenochaetaceae (order Hymenochaetales) and have annual poroid (polypore) fruiting bodies. They are found on trunks of living angiosperm trees or decaying wood. *I. levis* mainly inhabits trunks of cultivated *Platanus* and *Ulmus* trees [5]. While *I. cuticularis* is more or less widely distributed in the northern hemisphere, *I levis* is known in some countries in west- and central Asia and has only been recently recorded from Italy [5,6].

Medicinal properties of *I. cuticularis* have been shown, including hemostasis, antitumor, and free radical scavenging properties [7-9]. *I. levis* is known to have antitumor, diabetes and hypertriglyceridemia

treatment, and insulin-regulating properties [10-12]. Both species are included in Chinese medicinal mushrooms [13]. However, knowledge of their metabolites is lacking, and none of these fungi have been investigated regarding their chemical profile. In this study, we aim to determine the bioactivity of n-hexane, acetone, and ethyl acetate extracts of *I. levis* and *I. cuticularis* and investigate the metabolites of the two species by HPLC-DAD, GC-MS, and LC-ESI-MS/MS methods for the first time.

MATRIALS AND METHODS

Fungal Material and Fungal Extracts

Fungal samples were collected by the authors (SC and MG) in Iran and were identified by MG (Fig. 1). Part of the voucher samples was deposited in the Iranian Cryptogamic Herbarium (Index Herbarium acronym ICH) in Tehran. The remaining samples were powdered and used for metabolite extraction.

Fig. 1 In situ photographs of (a) *I. levis* and (b) *Inonotus cuticularis*. Photos by MG.

Fungal extraction was performed using the maceration method, followed by ultrasound-assisted extraction in an ultrasonic bath [14]. Thirty grams of fungal powder were extracted by n-hexane and filtered after 24 h. The dried residue was combined with acetone, sonicated in an ultrasonic bath for one h, and shacked for 48 h. Re-extracting by ethyl acetate was carried out after the refinement of residues. The extracts were stored at -40 ºC after filtration and complete drying. Hot water extraction from the fungi was performed based on [15].

Antimicrobial Tests

The antimicrobial activity of fungal extracts was evaluated against three gram-positive bacteria, including *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC25923, *Streptococcus mutans* ATCC 35665, and three gram-negative bacteria, including *Acinetobacter baumannii* BAA-744, *Escherichia coli* ATCC8739, and *Pseudomonas aeruginosa* ATCC9027, as well as against *Candida albicans* ATCC10231. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were assessed using broth micro-dilution by 96-well sterile plates. Serial dilutions of the extracts were prepared in Muller-Hinton broth and DMSO (5% v/v). The microbial suspensions were prepared according to McFarland's 0.5 and inoculated per well. The microplate was incubated at 37 ℃ for 24 h. The first well without turbidity defined the MIC. Ten µL of the extracts from wells with no turbidity were cultured at Muller-Hinton Agar and incubated at 37 ℃ for 24 h. The first concentration with no growth was MBC [16, 17].

DPPH and ABTS Antioxidant Assays

The antioxidant assay using 2,2-diphenyl 1-1-picrylhydrazyl (DPPH) was performed following [18], with some modifications. For this assay, 50 µL DPPH solution (100 µM) was added to 150 µl of different ascorbic acid concentrations, and each extract and the mixture were stored in the dark for 30 minutes. The absorbance was read by NanoDrop (Bio-Tek, epoch, USA) at 517 nm. For control, 100 µL of the extract was replaced with pure methanol. The percentage of inhibition of DPPH free radicals was calculated with the following equation:

$$AA\,(\%) = (A0 - A1)/A0 \times 100$$

Where AA stands for the percentage of inhibition, A0 is the absorbance of the control, and A1 stands for the absorbance of the sample. The anti-radical activity of the extracts was compared based on the IC_{50} parameter.

The antioxidant assay based on ABTS (2,20 -azino-bis (3- ethylbenzothiazoline-6-sulphonic acid) as cation radical was performed following [19]. Seven mM ABTS aqueous solution was oxidized by 2.45 mM potassium persulfate, and the resulting solution was stored in the dark. After 12–16 h, phosphate buffer was added, and the absorbance was set to 0.70 ± 0.05. The sample reaction was mixed with 0.07 mL of the extracts (with 1000, 500, 250, 125, 62.5 µg/mL concentrations) and 3 mL of the ABTS. Following six minutes of storage in the dark, the absorbance was read at 734 nm by the spectrophotometer. Trolox was used as a positive control. The percentage of inhibition was calculated with the same equation as above.

Total Phenolic and Total Polysaccharide Content

The total phenolic content of the fungal extracts was calculated using the Folin-Ciocalteu method following [20]. Twenty µL of extract (1 mg/mL) was mixed with 1.60 mL distilled water and 100 µL Folin-Ciocalteu reagent (1:10 v/v). After 1–8 min, 300 µL of sodium bicarbonate [7.5% (w/v)] was added. The absorbance of the samples was assessed at 760 nm after 30 min using a spectrophotometer (T80 + UV/Vis spectrometer- PG instrument Ltd). The results were reported as mg GAE/g in the dried extract.

Total polysaccharide content was measured using the phenol-sulphuric acid method [21], with D-glucose as the standard. Accordingly, 500 µL of hot water extract solution (0.1 mg/mL) was mixed with 500 µL phenol (5%), to which 2500 µL of Sulphuric acid was added. The absorbance of the reaction was read at 490 nm after one h. The amount of total polysaccharide was estimated based on the standard glucose calibration curve and reported on µg glucose in one mg of hot water extract.

Cytotoxicity Assay

The cytotoxicity effect of fungal extracts was evaluated on normal human dermal fibroblasts (HDF, NCBI Code: C645) and Hela C115 cell line based on an MTT assay using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl Tetrazolium bromide [22]. The cell lines were purchased from the Pasteur Institute of Iran and cultured following the instructions. The cells were loaded into a 96-well plate (104 cells per well) and treated with five different concentrations of the fungal extract. We used caffeic and gallic acid as the control for a phenolic compound according to their detection in HPLC-DAD analyses in acetonic extracts. Also, kanamycin, fluconazole, and cisplatin were used for drug control. After 24 h, the media was removed from the cells, and 100 µL of tetrazolium salt solution with a concentration of 0.5 mg/mL was added to each well and incubated for four h. Following the removal of the salt solution, 50 µl DMSO was added to each well. After 10 min incubation, the absorbance of each well was read by an ELISA reader (Bio-Tek, epoch, USA) at 570 nm. The cell viability percentage was measured with the following equation, and the IC_{50} values were calculated:

% inhibition of cell viability = (control Abs-sample Abs)/ (control Abs) × 100

HPLC-DAD Analysis

Analysis, identification, and quantification of 10 phenolic compounds were performed by HPLC Agilent 1200 (Santa Clara, Ca, USA). The instrument was composed of a gas vacuum cleaner, automatic sampling, a binary pump, and a Diode-Array Detection (DAD) system. Data analysis was performed using Agilent HPLC ChemStation software. The mobile phase consisted of distilled water and 1% acetic acid (A), and methanol (B), with a flow rate of 0.7 mL/min. The gradient program was as follows: 0–25 min, 10–22 % B and 90–78 %A; 25–60 min, 22–50% B and 78–50% A; 60–70 min, 50–95% B and 50–5% A. The experiment was set on the range of eight wavelengths and done for 70 minutes. The injected volume of samples was 10 µL. The temperature was 25 ºC. Ten phenolic compounds, namely gallic acid, resorcinol, chlorogenic acid, caffeic acid, syringic acid, vanillic acid, veratric acid, salicylic acid, p-coumaric acid, and ellagic acid, were used as standards. The quantification of detected compounds was performed using standard calibration curves.

LC-ESI-MS/MS Analysis

The LC-ESI-MS/MS analysis of fungal compounds was performed by the Waters Alliance e2695 model (Waters Corporation, Milford, MA, USA). The column was Atlantis T3 C18 (2.1 mm × 100 mm, 3 µm; Waters Corp., Milford, MA, USA), kept at 30 °C. MS spectra were obtained from Mass Spectrometer (Quattro micro API model) (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization

source (ESI). The fungal extracts were prepared by dissolving 10 mg of the acetonic fungal extract in 10 mL of HPLC-grade methanol.

The elution process was performed with acetonitrile combined with 1% formic acid (A) and water mixed with 1% formic acid (B) within 20 minutes, according to the existing elution program: (5% eluent A, 95% eluent B) in 0–10 minutes, then (95% eluent A, 5% eluent B) in 10–20 minutes. The injection volume of the extract was five µL, and the other MS parameters were optimized as follows: source temperature 300 ℃, capillary voltage 3 kV, and cone voltage 20 V. Nitrogen was sprayed for drying and precipitation. The collision energy selected for the decomposition of MS/MS was 30eV [23]. The data were processed utilizing the Waters software MassLynx V4.1. In addition, the PubChem and MassBank databases and various published articles were consulted for tentative compound identification.

GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on an Agilent 6890 series and Mass spectrometer model Agilent 5973 to investigate the compounds in the n-hexane extract of *I. levis* and *I. cuticularis*. This device contained a selective mass detector, GC equipped with a Varian VF-1ms column with 30 meters' length, 0.25 mm inner diameter, and 0.5 micrometers film thickness. The injector temperature was 280 ℃, and the splitting ratio was 1:100. The injection volume was one µL, and the helium gas (99.999%) was used with a constant flow rate of 1 mL/min. The temperatures of the oven system were as the following: oven temperature program 80 °C at the rate of 0.0 (C/min)-2 min hold, up to 280 °C at the rate of 10 ° (C/min)-20 min hold, injector temperature 280 ℃, total running time 42 min. The MS quadrupole analyzer was in the electron-influenced ionization mode (EI) with an energy of 70 eV with a solvent delay of 3 minutes, in the range of 40–400 m/z, with a source temperature of 230 °C. The data was analyzed using the NIST MS spectral program and the Wiley mass spectral library [24].

Statistical Analysis

All experiments were done in triplicate. The mean of treatments was compared using one-way and two-way ANOVA. All statistical analyses were performed using SPSS software version 16 at a $p < 0.05$.

RESULTS AND DISCUSSION

Antimicrobial Activity

The results of antibacterial and antifungal assays are presented in Table 1. The results show that the extracts of *I. cuticularis* and *I. levis* have antibacterial properties, but the effect varies depending on the extracts and bacterial strains. The antibacterial activity of *I. cuticularis* acetonic extract was higher than other extracts, with MIC values ranging from 12.5 to 100 mg/mL. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were more resistant than the other bacteria. Hot water extract of *I. levis* showed no antimicrobial effect, but hot water extract of *I. cuticularis* indicated antimicrobial activity against *S. aureus, S. mutans,* and *C. albicans* with a MIC of 100 mg/mL. *Streptococcus mutans* was more sensitive than other bacteria to all extracts. As expected, the gram-positive bacteria were more susceptible to extracts than the gram-negative bacteria. Liu et al. also showed that the extracts of *I. sanghuang* have more potent antibacterial activity on gram-positive bacteria [25]. In addition, *I. linteus* fruiting body extracts showed antimicrobial activity against methicillin-resistant *S. aureus* [26]. The outer membrane of gram-negative bacteria is the main reason for resistance to a wide range of antibiotics. Some modifications in the membrane may lead to resistance, such as changing the hydrophobic properties or mutations in porins [27, 28].

Tamrakar et al. [29] reported the antibacterial activity of ethanol extracts of 90 Nepalese wild mushrooms against *S. aureus* and *Propionibacterium acnes*. They determined that Hymenochaetales was the strongest fungal group, with samples showing more than 80% inhibition of *S. aureus*. The highest inhibitory effect was observed for *I. clemensiae*, *I. andersonii*, and *I. cuticularis* with an MBC of 100 µg/mL.

In our study, ethyl acetate, acetone, and hot water extracts of *I. cuticularis* showed weak antifungal activity against *Candida albicans*, but *I. levis* did not affect this yeast. According to Angelini et al. [30], *I. hispidus* extracts have antifungal properties against *C. albicans.*

Antioxidant Activity

DPPH and ABTS assays showed that our fungal extracts have moderate to high antioxidant activity (Table 2).

Table 1 Antimicrobial activities of *I. levis* and *Inonotus cuticularis* extracts expressed as MIC and MBC values (mg/mL).

Bacteria	*A. baumannii*		*E. coli*		*P. aeruginosa*		*B. subtilis*		*S. aureus*		*S. mutans*		*C. albicans*	
Extracts	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
AEIL	100±00	ND	50±00	ND	100±00	ND	50±00	100±00	25±00	100±00	25±00	50±00	ND	ND
EEIL	ND	ND	100±00	ND	ND	ND	50±00	ND	50±00	ND	50±00	100±00	ND	ND
HEIL	ND	ND	ND	ND	ND	ND	100±00	ND	100±00	ND	50±00	ND	ND	ND
HWEIL	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AEIC	50±00	ND	25±00	50±00	100±00	ND	25±00	50±00	12.5±00	50±00	12.5±00	25±00	100±00	ND
EEIC	100±00	ND	50±00	ND	ND	ND	50±00	100±00	50±00	100±00	25±00	50±00	100±00	ND
HEIC	ND	ND	ND	ND	ND	ND	100±00	ND	50±00	ND	50±00	100±00	ND	ND
HWEIC	ND	ND	ND	ND	ND	ND	ND	ND	100±00	ND	100±00	ND	100±00	ND
Kanamycin	0.01±00	0.03±00	0.004±00	0.009±00	0.625±00	1.25±00	0.001±00	0.002±00	0.07±00	0.156±00	0.003±00	0.007±00	ND	ND
Fluconazole	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.001±00	0.007±00

AEIL: Acetonic extract of *I. levis*. EEIL: Ethyl acetate extract of *I. levis*, HEIL: n-Hexane extract of *I. levis*. HWEIL: Hot water extract of *I. levis*. AEIC: Acetonic extract of *I. cuticularis*. EEIC: Ethyl acetate extract of *I. cuticularis*. HEIC: n-Hexane extract of *I. cuticularis*, HWEIC: Hot water extract of *I. cuticularis.*

Table 2 Total phenol and total polysaccharide content in *I. levis* and *I. cuticularis*, and IC_{50} values for antioxidant assays.

Extract	Total phenol (mg GAE/g)	Total polysaccharide (µg/mg)	IC_{50} (µg/mL)	
-	-	-	DPPH	ABTS
AEIL	35 ± 1.8	ND	355.34	222.12
EEIL	25.5 ± 1.5	ND	469.02	239.78
HEIL	ND	ND	556.23	331.52
HWEIL	ND	174.83 ± 8.08	385.44	311.50
AEIC	92.16 ± 1.04	ND	144.98	128.06
EEIC	68 ± 1	ND	191.44	158.41
HEIC	ND	ND	352.66	271.31
HWEIC	ND	325.92 ± 5.09	333.52	290.23
Ascorbic acid	-	-	4.94	-
Trolox	-	-	-	22.36

AEIL: Acetonic extract of *I. levis*. EEIL: Ethyl acetate extract of *I. levis*, HEIL: n-Hexane extract of *I. levis*. HWEIL: Hot water extract of *I. levis*. AEIC: Acetonic extract of *I. cuticularis*. EEIC: Ethyl acetate extract of *I. cuticularis*. HEIC: n-Hexane extract of *I. cuticularis*, HWEIC: Hot water extract of *I. cuticularis*, ND: not determined.

Table 3 The IC_{50} values of *I. levis* and *I. cuticularis* extracts on normal HDF and on HeLa cells.

	HDF cell line	HeLa cell line
Fungal extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)
AEIL	1447	731
HWEIL	1673	551
AEIC	1760	247
HWEIC	1908	82
Kanamycin	>100	ND
Fluconazole	>100	ND
Cisplatin	93	29
Caffeic acid	>100	>100
Gallic acid	>100	>100

AEIC: Acetonic extract of *I. cuticularis*, AEIL: Acetonic extract of *I. levis*, HWEIC: Hot water extract of *I. cuticularis*, HWEIL: Hot water extract of *I. levis*.

Table 4 Phenolic compounds in *I. levis* and *I. cuticularis* detected by HPLC-DAD.

Standard compounds	T_R (min)	R^2	Wavelength (nm)	Amount (µg/mL)			
				AEIL	EEIL	AEIC	EEIC
Caffeic acid	24.9	0.99979	330	5	<5	5.8	<5
Chlorogenic acid	21.9	0.99952	330	ND	<5	ND	ND
Ellagic acid	53.0	0.99586	254	ND	ND	<5	ND
Gallic acid	5.4	0.99978	278	49.9	40	24.6	ND
p-Coumaric acid	45.2	1.00000	300	ND	ND	<5	ND
Resorcinol	8.9	0.99975	278	<5	ND	ND	ND
Salicylic acid	44.2	0.99977	278	ND	<5	ND	<5
Syringic acid	29.6	0.99981	278	ND	ND	ND	ND
Vanillic acid	30.7	0.99976	260	<5	<5	ND	<5
Veratric acid	42.3	0.99975	260	ND	<5	ND	<5

AEIL: Acetonic extract of *I. levis*. EEIL: Ethyl acetate extract of *I. levis*, AEIC: Acetonic extract of *I. cuticularis*. EEIC: Ethyl acetate extract of *I. cuticularis*, ND: not detected.

The acetonic and ethyl acetate extracts had more potent antioxidant activity than n-hexane extracts. The IC_{50} values for the ABTS and DPPH assay ranged from 144.98 to 556.23 µg/mL and 128.06 to 331.52 µg/mL, respectively. Significant differences ($p < 0.05$) were observed between the tested extracts. The highest antioxidant activity was shown by the acetonic extract of *I. cuticularis*, with an IC_{50} value of 144.98 µg/mL in DPPH and 128.06 µg/mL in ABTS assays. The n-hexane extract of *I. levis* showed the weakest antioxidant activity, with an IC_{50} value of 556.23 µg/mL and 331.52 µg/mL in DPPH and ABTS assays, respectively.

Total Phenolic and Polysaccharide Content

The total phenolic content of the fungal extracts is reported in Table 2. The phenolic content in acetonic extract ranged from 25.50 to 92.16 mg GAE/g. The acetonic extract of *I. cuticularis* showed the highest total phenolic content (92.16 mg GAE/g). The total phenolic content of ethyl acetate and acetonic extracts ranged from 25.50 to 35.00 mg GAE/g for *I. levis* extracts and 68.00 to 92.16 mg GAE/g for *I. cuticularis*. Moreover, in our previous study, the phenolic content of *I. levis* ethanolic extract contained 17.50 mg GAE/g [14]. On the other hand, Liu et al. [25] reported the total phenolic content of *I. sanghuang* ranging from 0.79 to 43.60 mg GAE/g. The total polysaccharide content of the fungal extracts is shown in Table 2. The total polysaccharide content in *I. cuticularis* and *I. levis* measured 328.58 and 174.83 µg glucose in one mg of hot water extract, respectively ($p < 0.05$).

Cytotoxicity Assay

The cytotoxicity effect of fungal extracts on HDF cells is reported in Table 3 (see also Fig. 2). The highest IC_{50} value (1908 µg/mL) was expressed by the hot water extract of *I. cuticularis*. We determined the IC_{50} value of acetonic and hot water extracts of *I. cuticularis* and *I. levis* on HDF normal cell line to be approximately 1447 µg/mL to 1908 µg/m L. The extracts in lower doses showed low cytotoxicity on normal HDF cells. According to

other reports, the polypore fungi *Fomitopsis betulina* and some *Inonotus* species had no cytotoxicity on normal skin fibroblasts [31]. Also, our previous study noted the IC_{50} of *Phellinus tuberculosus* and *Fuscoporia ferruginosa* extracts were 2020 µg/mL and 1410 µg/mL, respectively, on HDF normal cells [23]. The IC_{50} value of hot water and acetonic extracts of *I. cuticularis* (82 and 247 µg/mL, respectively) on the HeLa cell line was more potent than that of *I. levis* extracts (731 and 551 µg/mL). Song et al. [32] reported that polysaccharides of *Inonotus obliquus* inhibited the proliferation of cervical HeLa cancer cells. In this study, caffeic acid, gallic acid, fluconazole, and kanamycin had no cytotoxic effect on HDF and HeLa cell line in the selected concentrations. The concentrations of phenolic compounds were chosen based on the amount detected in HPLC analysis, and the concentrations of drugs were determined based on their MIC values. The IC_{50} values of cisplatin on HDF normal and HeLa cells were 93 and 29 µg/mL, respectively.

HPLC-DAD Analysis

The results of the HPLC-DAD analysis are reported in Table 4. Gallic acid had the highest amount, with 49.9 µg/mL and 40 µg/mL in the acetonic and ethyl acetate extract of *I. levis*, respectively, and 24.6 µg/mL in the acetonic extract of *I. cuticularis*. Caffeic acid was also seen in the acetonic extract of *I. cuticularis* and *I. levis* at 5.8 and 5 µg/mL, respectively. Some compounds such as resorcinol, chlorogenic acid, vanillic acid, veratric acid, salicylic acid, p-coumaric acid, and ellagic acid were < 5 µg/mL or not determined in some extracts. Our results show that the concentration of gallic acid for *I. levis* is higher than *I. cuticularis*. Rajamanickam et al. [33] reported the antibacterial activity of gallic acid. Other compounds, such as caffeic acid, have antimicrobial and antioxidant effects and have been detected in other polypore fungi such as *Inonotus hispidus*, *Fuscoporia ferruginosa*, *Phellinus tuberculosus*, and *Fomes fomentarius* [22, 23, 30, 34].

LC-ESI-MS/MS Analysis

The results of the proposed compounds in the LC-ESI-MS/MS are reported in Table 5. The mass spectrum obtained from the analysis suggested a preliminary identification of 18 compounds for *I. levis* and 17 compounds for *I. cuticularis* (Table 5). Moreover, several compounds detected by our HPLC-DAD analyses were also detected here. In general, styrylpyrones, furans, sesquiterpenoids, and hispidin derivatives were detected in both fungi. These compounds are known to have meaningful biological activities, including antimicrobial, antioxidant, and anticancer properties, e.g. [29,35]. Compounds common to both fungal species include inotilone, inonotin H and C, phellinulin B and M, cinnamic acid, p-coumaric acid, caffeic acid, phelligridin A and D, hispidin, and gallic acid.

Inotilone (an unusual 5-methyl-3(2H)-furanone derivative) is a phenylpropanoid derived polyketide, also reported from *Inonotus hispidus,* and has shown anticancer, immunomodulatory, and antiviral activities [15, 36]. Inonotusic acid is a diterpene discovered by Liu et al. [37] from *I. obliquus* with hepatoprotective effects. In our study, inonotusic acid was detected in *I. cuticularis*. Inonotsudiol A is a lanostane-type triterpenoid first isolated from the sclerotia of *Inonotus obliquus* with moderate anticancer activity [38]. This compound was detected here in *I. cuticularis*. Daedalin A is a benzopyran (chromene) derivative first described from *Daedalea dickinsii* (Fmitopsidaceae) by Morimura et al. [39], showing tyrosinase inhibitory and antioxidant activities [40]. Here, we report daedalin A for the first time from the family Hymenochaetaceae in *I. cuticularis*.

Ribisin B, a derivative of benzofuran, detected here in *I. levis*, has been first isolated from *Phellinus ribis* (current name *Phylloporia ribis*) by Liu et al. [41] with anti-Alzheimer activity. The hispidin derivative phaeolschidin E, described from *Phaeolus schweinitzii* (Laetiporaceae) [42], was detected here in *I. levis*. Our previous study reported Phaeolschidin A–C in *Phellinus tuberculosus*. The compounds phellinulin, phellifuropyranone A, phelligridin, hispidin, baumin, caffeic acid, and gallic acid, detected in the present study in *I. levis* and *I. cuticularis*, have also been noticed in *Fuscoporia ferruginosa* and *Phellinus tuberculosus* in our previous study and were thoroughly discussed there in [23].

Table 5 Detected compounds in *I. levis* and *I. cuticularis* by LC-ESI-MS/MS in negative mode.

Tentative identification	Molecular formula	Molecular mass (g/mol)	$[M-H]^-$ (*m/z*)*	MS2 Fragments (*m/z*) %	*I. levis*	*I. cuticularis*	Ref.
Baumin	$C_{27}H_{22}O_{11}$	522.5	521.481	-	-	+	[43]
Caffeic acid	$C_9H_8O_4$	180.16	179.274	-	+	+	[44]
Chavicol	$C_9H_{10}O$	134.18	132.98 (133)	43 (23), 59 (20), 70 (100), 73 (30)	+	-	[45]
Cinnamic acid	$C_9H_8O_2$	148.16	147.006	73 (100), 43 (40), 85 (30)	+	+	[46]
P-Coumaric acid	$C_9H_8O_3$	164.16	163.062	-	+	+	[47]
Daedalin A	$C_{11}H_{12}O_3$	192.214	191	-	-	+	[40]
3,4-dihydroxybenzalacetone	$C_{10}H_{10}O_3$	178.18	177.24	133 (100), 159 (20)	-	+	[48]
Gallic acid	$C_7H_6O_5$	170.12	169.13	-	+	+	[49]
3-Hexyloxy -4-Hydroxybenzaldehyde	$C_{13}H_{18}O_3$	222.28	221	59 (100), 71 (60), 85 (57)	+	-	[50]
4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.12	120.97 (121)	92 (100), 121 (30)	+	-	[51]
Hispidin	$C_{13}H_{10}O_5$	246.21	245.234	82 (36), 95 (53),109 (77),130 (100)	+	+	[52]
Inonotin C	$C_{15}H_{24}O_3$	252.35	251.36	80 (100),79 (95),81 (80),89 (10)	+	+	[53]
Inonotin H	$C_{15}H_{26}O_2$	238.37	237.182	45 (100), 73 (40), 176 (25)	+	+	[53]
Inotilone	$C_{12}H_{10}O_4$	218.2	217.386	35 (100), 59 (18), 101 (57)	+	+	[54]
Inonotusic acid	$C_{21}H_{28}O_2$	312.4	311.482	-	-	+	[37]
Inonotsudiol A	$C_{30}H_{50}O_2$	442.7	441	-	-	+	[38]
Phaeolschidin E	$C_{17}H_{15}NO_6$	329.3	328.28	113 (70), 188 (100)	+	-	[42]
Phellifuropyranone A	$C_{21}H_{14}O_7$	377.347	377.347	35 (100), 73 (25), 89 (30)	-	+	[55]
Phellinulin B	$C_{15}H_{20}O_2$	232.32	231.1	71 (100), 98 (30), 146 (40)	+	+	[56]
Phellinulin M	$C_{15}H_{26}O_2$	238.37	237.251	79 (100), 80 (30), 112 (17) 136 (20),	+	-	[56]
Phelligridin A	$C_{13}H_8O_6$	260.2	259.422	109 (47), 124 (100), 138 (42), 166 (60)	+	-	[57]
Phelligridin D	$C_{20}H_{12}O_8$	380.3	379.53	-	-	+	[57]
1-Phenylhept-3-En-4-One	$C_{13}H_{16}O$	188.26	187.301	134 (100),161 (14)	+	+	[58]
1-Phenylheptane-1,5-dione	$C_{15}H_{20}O_2$	205	203.513	116 (100), 74 (60), 142 (25)	+	-	[59]
Ribisin B	$C_{14}H_{14}O_5$	262.261	250.56	84 (48), 109 (100), 166 (65), 184 (25)	+	-	[41]

*The ion obtained by abstraction of the proton from the analyte molecule.

Table 6 Detected compounds in *I. levis* and *I. cuticularis* by GC-MS.

Tentative identification	Molecular formula	Molecular mass (g/mol)	*I. levis*	*I. cuticularis*
Benzenemethanamine, N- (4-chlorophenyl)-N- (phenylmethyl)-	$C_{20}H_{18}ClN$	307.8	+	-
Cyclohexane, 1-ethyl-1-methyl-	C_9H_{18}	126.2392	+	-
1-Decene, 4-methyl-	$C_{11}H_{22}$	154.2924	-	+
3-Decen-5-one, 2-methyl-	$C_{11}H_{20}O$	168.2759	+	+
Docosane	$C_{22}H_{46}$	310.6006	-	+
Dodecane	$C_{12}H_{26}$	170.3348	-	+
Eicosane	$C_{20}H_{42}$	282.5475	-	+
Ethyl linoleate	$C_{20}H_{36}O_2$	308.4986	+	-
1-Ethyl-1-methylcyclohexane	C_9H_{18}	126.24	-	+
1-Ethyl-2-methylcyclohexane	C_9H_{18}	126.24	+	-
Ethyl oleate	$C_{20}H_{38}O_2$	310.5145	+	-
Ergosta-7,22-dien-3-ol	$C_{28}H_{46}O$	398.7	+	-
Ergosta-5,7,22-trien-3-ol	$C_{28}H_{44}O$	396.6	+	+
2-Furanglycolic acid	C6H6O4	142.11	-	+
Heneicosane	$C_{21}H_{44}$	296.5741	-	+
Heptadecane	$C_{17}H_{36}$	240.4677	-	+
Hexacosane	$C_{26}H_{54}$	366.7070	-	+
Hexadecane	$C_{16}H_{34}$	226.4412	-	+
Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4241	+	-
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.4772	+	-
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	+	+
Linoleic acid	$C_{18}H_{32}O_2$	280.4455	+	+
7-Methoxy-2-methylisoquinoline-3,5,8-trione	$C_{11}H_9NO_4$	219.19	-	+
Nonadecane	$C_{19}H_{40}$	268.5209	-	+
Octadecane	$C_{18}H_{38}$	254.4943	-	+
Octadecane, 3-ethyl-5- (2-ethylbutyl)-	$C_{26}H_{54}$	366.7	-	+
8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.501	+	+
9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.4879	+	+
9,17-Octadecadienal, (Z)-	$C_{18}H_{32}O$	264.4461	-	+
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.4455	+	-
Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.5038	+	+
Pentacosane	$C_{25}H_{52}$	352.6804	-	+
Tricosane	$C_{23}H_{48}$	324.6272	-	+
Tridecane	$C_{13}H_{28}$	184.3614	-	+
Tetradecane	$C_{14}H_{30}$	198.3880	-	+
Tetracosane	$C_{24}H_{50}$	338.6538	-	+
Undecane	$C_{11}H_{24}$	156.3083	-	+

GC-MS Analysis

The GC-MS analyses showed 16 compounds in *I. levis* and 28 in *I. cuticularis* (Table 6). Compounds detected in both fungi included ergosta-5,7,22-trien-3-ol, linoleic acid, oleic acid (9-Octadecenoic acid Z), hexadecanoic acid methyl ester, 3-decen-5-one, 2-methyl-, 8,11-octadecadienoic acid methyl ester, and octadecanoic acid methyl ester. The fatty acids linoleic acid, oleic acid, and hexadecanoic acid (palmitic acid) have also been found in *Inonotus obliquus*, *Phellinus linteus*, and some other *Phellinus* species [60-62]. Fatty acids play vital roles in several metabolic pathways inside the human body. The antibacterial effect of ergosta-5,7,22-trien-3-ol was reported by Vazirian et al. [63] isolated from an n-hexane extract of the polypore fungus *Ganoderma lucidum*.

Ergosta-7,22-dien-3-ol is one of the ergosterol derivatives and was found in *I. levis* in the present study. Ergosterol is a vitamin D precursor converted by UV light into ergocalciferol which is considered a natural antitumor [64-66]. Sun et al. [67] and Cui et al. [15] found that the ethanol extracts of *Inonotus obliquus*, containing inotodiol and ergosterol peroxide, had strong antioxidant properties. Kusumah et al. [68] reported the antibacterial effects of linoleic acid, octadecanoic acid methyl esters, and ethyl linoleate. Volatile compounds, such as hydrocarbons, alcohols, fatty acids, ketones, and esters, have medicinal properties [69,70].

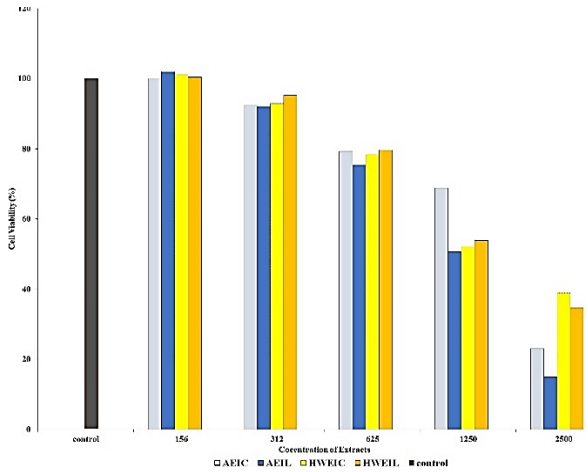

Fig. 2 Cell viability percentage of *I. levis* and *Inonotus cuticularis* extracts on HDF normal cells after 24 h MTT assay. AEIC: Acetonic extract of *I. cuticularis*, AEIL: Acetonic extract of *I. levis*, HWEIC: Hot water extract of *I. cuticularis*, HWEIL: Hot water extract of *I. levis*.

CONCLUSIONS

This study provided the first results on the chemical profiling of the two polypore basidiomycete fungi, *I. levis* and *I. cuticularis*, using HPLC-DAD, LC-ESI-MS/MS, and GC-MS methods. The tentatively detected compounds comprise a range of diverse phenolic, sesquiterpenoids, furans, and volatile compounds, many of which with established biological activities. According to the results, Inonotus cuticularis exhibited more potent antibacterial and anticancer effects. We also showed that the acetonic extracts of the two medicinal polypore fungi I. levis and I. cuticularis have noticeable antibacterial activities, though the anti-Candida effects were generally low. Moreover, we showed that our fungal extracts have moderate to high antioxidant activity. Therefore, I. levis and I. cuticularis display dual antioxidant and antibacterial properties. We suggest that I. levis and I. cuticularis warrant thorough research on their bioactive metabolites characterization and purification and could potentially open new windows in mycochemistry, natural product-based drug discovery, and medicin.

Author Contributions

SCD and MG collected the fungal samples, developed the original idea, and wrote the manuscript; SCD performed the experiments; MM finalized the draft; SCD and HN analyzed the chemical data. All authors read the draft and commented on it.

Statements and Declarations

Consent to participate: All authors read and agreed on the final draft.

Conflict of interest: The authors declare no competing interests.

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