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Impact of Different Extraction Methods on the *In Vitro* Bioactivity Profiles of *Terfezia Claveryi*

Guney Gursoy[1], **Kubra Ozturk**[2], **Zehra Cicek**[3], **Esin Kiray**[4], **Cigdem Er Caliskan**[5]

[1]Department of Biophysics, Department of Basic Medical Sciences, Faculty of Medicine, Kirsehir Ahi Evran University [2]Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Kirsehir Ahi Evran University [3]Department of Physiology, Gulhane Faculty of Medicine, University of Health Sciences [4]Department of Medical Services and Techniques, Medical Laboratory Techniques Program, Vocational School of Health Services, Kirsehir Ahi Evran University [5]Department of Field Crops, Faculty of Agriculture, Kirsehir Ahi Evran University

Corresponding Author : Guney Gursoy <guneygursoy@ahievran.edu.tr>

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Abstract

Terfezia claveryi is an edible desert truffle traditionally consumed in arid regions. In recent years, it has attracted increasing scientific interest due to its potential antioxidant, antimicrobial, and therapeutic properties, suggesting possible applications in food and health-related fields. However, despite growing evidence of its biological potential, the influence of different extraction strategies on the recovery of bioactive compounds and the resulting *in vitro* bioactivity profile of *Terfezia claveryi* has not yet been clarified in the literature. In this study, the *in vitro* bioactivity of *Terfezia claveryi* extracts obtained using different extraction methods and solvents with varying polarity was evaluated. Extraction yield was determined, and the obtained extracts were assessed for antioxidant, antimicrobial, antibiofilm, and cytotoxic activities using established testing systems. Antioxidant capacity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) tests, while cytotoxic effects were investigated in SH-SY5Y neuroblastoma cells. Extraction yield varied depending on both method and solvent polarity, with the highest yield obtained from the aqueous extract prepared by the ultrasound-assisted extraction (UAE) method (18.40%). It was determined that antioxidant activity showed significant differences depending on the extraction method, and aqueous extracts obtained by the maceration-assisted extraction (MAE) method exhibited a profile rich in phenolic compounds along with the strongest radical scavenging capacity. LC-MS/MS analysis of this extract revealed the presence of nine phenolic compounds; quinic acid and chlorogenic acid were identified as the dominant components. In contrast, Soxhlet-derived extracts induced greater cytotoxic effects in SH-SY5Y cells. Antimicrobial and antibiofilm responses displayed relatively limited variation among extraction methods, indicating a weaker dependence on extraction strategy for these activities. Overall, these findings demonstrate that the biophysical extraction method and solvent selection are critical determinants of the *in vitro* bioactivity profile of *Terfezia claveryi* extracts, with pronounced effects observed for antioxidant and cytotoxic activities.

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Introduction

The use of various mushroom species to improve health and to prevent and treat diseases dates back to ancient times[1,2,3]. Macrofungi are generally classified into two groups: Epigeous (mushrooms) and hypogeous (truffles)[4]. Known as the desert truffle, *Terfezia claveryi* (*T. claveryi*) is a world-renowned and edible truffle species belonging to the genus *Terfezia*[5]. *T. claveryi* is commonly distributed in arid and semi-arid regions where *Helianthemum* species are prevalent[6,7,8,9,10,11]. In recent years, the global burden of chronic diseases such as cancer and neurodegenerative disorders has increased significantly, highlighting the urgent need for new, safe, and effective therapeutic agents. According to global health reports, cancer remains one of the leading causes of death worldwide, while neurodegenerative diseases continue to increase alongside an ageing population. Given this growing public health issue, the investigation of natural products derived from fungi and medicinal plants as potential sources of bioactive compounds has become an important research priority[12].

In parallel, the rapid emergence of antimicrobial resistance poses another major threat to global health. Drug resistance has significantly reduced the effectiveness of traditional antibiotics, creating an urgent need for alternative antimicrobial strategies[13]. Secondary metabolites derived from natural sources, particularly phenolic and polyphenolic compounds, are attracting increasing interest due to their diverse antimicrobial mechanisms and low tendency to develop resistance[14]. Oxidative stress is a fundamental mechanistic factor underlying many chronic diseases, including cancer, neurodegenerative disorders, and metabolic syndromes. Excessive production of reactive oxygen species (ROS) can ultimately disrupt cellular homeostasis by causing DNA damage, lipid peroxidation, and protein oxidation. In this context, natural antioxidants that can scavenge free radicals and regulate redox balance are considered promising agents for mitigating oxidative damage and disease progression[15].

Truffles are rich in unsaturated fatty acids, proteins, minerals, vitamins, amino acids, phenolics, and polyphenols, components that have been associated with antimicrobial, antioxidant, and anticancer properties[16,17,18,19,20,21,22,23,24]. Their antioxidant capacity has been linked to their effectiveness in scavenging free radicals[25,26,27]. Among truffle species, *T. claveryi* is particularly noteworthy due to its edible structure, regional economic value, and reported richness in phenolic compounds[23]. Despite emerging evidence regarding its traditional consumption and biological activity, comprehensive studies investigating how extraction strategies affect its chemical composition and biological effects remain limited.

The recovery of biologically active compounds from truffles is strongly influenced by extraction parameters, including the applied extraction technique and solvent polarity. Biophysical extraction methods such as ultrasound-assisted extraction (UAE), maceration-assisted extraction (MAE), and Soxhlet extraction (SE) show significant differences in terms of energy input, extraction efficiency, and selectivity for polar compounds[28]. However, most research has relied on a single extraction method or limited solvent systems, and studies that comparatively address the effects of these methods on the *in vitro* biological activity profile of *T. claveryi* are limited. Therefore, this study systematically compares multiple biophysical extraction methods using solvents of varying polarity to evaluate the *in vitro* biological activities of *T. claveryi* extracts. It was hypothesized that the extraction method and solvent polarity would lead to method-dependent differences in biological activities. Accordingly, the aims of the study are: (i) to compare extraction yields between different methods and solvents, (ii) to evaluate method-dependent variation in antioxidant and cytotoxic activities, and (iii) to examine antimicrobial and antibiofilm responses, considering their sensitivity to the extraction strategy.

Protocol

NOTE: The overall experimental workflow for the preparation and analysis of *T. claveryi* extracts is schematically summarized in **Figure 1**. All materials used in the study are described in the **Table of Materials**.

1. Fungal material authentication and preparation

1. Collection and processing of material

   NOTE: Fresh *T.claveryi* truffles were collected in May 2023 from steppe and sandy, uncultivated (non-agricultural) lands in Kırsehir Province, Central Anatolia, Türkiye. Sampling was carried out at three distinct locations: Dedeli Village (38°54'15.8" N, 34°06'40.3" E; ~1050 m above sea level), Buyukkayapa Village (38°53'45.1" N, 34°19'37.5" E; ~1020 m above sea level), and Akcaagıl Village (39°01'39.9" N, 34°12'39.8" E; ~1100 m above sea level). The collection sites are characterized by a semi-arid continental climate, with cold winters, hot and dry summers, and an average annual precipitation of approximately 380–420 mm. All samples were harvested during the natural fruiting season from areas with no recent agricultural activity to minimize potential anthropogenic contamination.

   1. Select clean and undamaged truffles, peel them, and wash three times with distilled water.
   2. Slice the samples into small pieces and shade-dry until a constant weight is achieved (approximately 20 days).
   3. Pulverize the dried material using a mechanical grinder and pass through a 328-micron (50 mesh) sieve.

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4. Store the processed samples in zip-lock bags in a cool, dry, and dark environment at room temperature until further analyses.

2. Molecular identification of *T. claveryi*

    1. Isolate genomic DNA from dried *T. claveryi* samples for molecular identification.
    2. Assess the DNA quantity and purity spectrophotometrically.
    3. Perform species identification polymerase chain reaction (PCR) by amplifying the internal transcribed spacer (ITS) region using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').
    4. Resolve PCR amplification products by electrophoresis on a 1.5% agarose gel prepared in 1x TAE buffer and visualized under UV illumination following ethidium bromide staining.
    5. Purify single-band PCR products according to the manufacturer's instructions[29]. Subject purified amplicons to Sanger sequencing.
    6. Assemble forward and reverse sequence reads into consensus sequences using the contig assembly programme (CAP) algorithm.
    7. Species identity was confirmed by comparison of the consensus ITS sequence with reference sequences available in public databases.

        NOTE: A voucher specimen of *T. claveryi* was retained in the laboratory collection for future reference. Species identification was confirmed by ITS-based molecular analysis (**Supplementary File 1**).

2. Fungal material preparation and extraction

1. Preparation of *T. Claveryi* extracts

    1. Weigh approximately 30 g of dried *T. claveryi* powder.
    2. Add 150 mL of solvent (methanol, acetone, n-hexane, ethyl acetate, or distilled water) to maintain a constant solid–liquid ratio across all extraction methods.
    3. Use analytical-grade solvents for all extraction procedures.
    4. Filter each extract under reduced pressure using qualitative filter paper.
    5. Concentrate the filtrates under reduced pressure at 30–45 °C using a rotary evaporator.
    6. Freeze-dry (lyophilize) the concentrated extracts until complete solvent removal is achieved.
    7. Store the dried crude extracts at −20 °C until further analysis.

        NOTE: Apply identical post-extraction processing steps to all samples to ensure methodological consistency and comparability.

2. Biophysical extraction methods

    1. Maceration-assisted extraction (MAE)

        1. Place the powdered *T. claveryi* sample into a closed extraction container.
        2. Add the selected solvent while maintaining a constant solvent-to-solid ratio.
        3. Incubate the mixture at room temperature under dark conditions for 72 h.
        4. Keep the containers closed to prevent solvent evaporation.
        5. Do not apply external agitation during the maceration period.
        6. Allow the mixture to settle at the end of the extraction period.
        7. Filter the extract prior to further processing.

    2. Soxhlet extraction (SE)

        NOTE: Ensure proper condenser water circulation and avoid dry heating during reflux.

        1. Place the powdered sample into a cellulose extraction thimble.
        2. Position the thimble inside the Soxhlet extraction chamber connected to a round-bottom flask.
        3. Add the selected solvent to the flask while maintaining a constant solvent-to-solid ratio.
        4. Heat the solvent to reflux using a thermostatically controlled heating mantle.
        5. Maintain continuous extraction for 24 h.
        6. Maintain the extraction temperature at the boiling point of each solvent (n-hexane: 68.7 °C; ethyl acetate: 77.1 °C; methanol: 64.7 °C; acetone: 56 °C; distilled water: 100 °C).

    3. Ultrasound-assisted extraction (UAE)

        NOTE: Monitor temperature continuously to prevent overheating during sonication.

        1. Place the powdered *T. claveryi* sample into the extraction vessel.
        2. Add the selected solvent while maintaining a constant solvent-to-solid ratio.

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3. Sonicate the mixture at a fixed frequency of 35 kHz and an output power of 520 W for 25 min.
4. Maintain the extraction temperature at approximately 30 °C.
5. Add ice at 5-minute intervals to keep the temperature within 25–28 °C during sonication.

3. Extraction efficiency
   1. Weigh the dried fungal material (W_p) prior to extraction.
   2. Weigh the dried crude extract (W_{ext}) after complete solvent removal.
   3. Calculate the extraction efficiency using Equation (1).

$$\text{Extraction efficiency}(\%) = \frac{W_{ext}}{W_P} \text{x } 100 \quad (1)$$

   4. Re-dissolve each dried crude extract in its respective solvent to prepare a stock solution at a standardized concentration of 10 mg/mL.
   5. Prepare stock solutions freshly prior to each *in vitro* bioactivity experiment.

      NOTE: Standardize all extract concentrations to ensure comparable dosing across subsequent bioactivity assays.

3. Antioxidant activity determination

1. Perform antioxidant activity analysis using DPPH, ABTS, and FRAP assays.
2. Prepare five different extract concentrations ranging from 0.25 to 1.0 mg/mL from freshly prepared stock solutions.
3. Conduct all measurements in triplicate to ensure reproducibility.
4. Record absorbance values spectrophotometrically using a microplate reader.

   NOTE: Perform all assays according to established literature protocols with minor modifications.

5. DPPH radical scavenging activity

   NOTE: The DPPH radical scavenging activity of *T. claveryi* extracts was evaluated according to the method described in the literature, with minor modifications[30]. Protect DPPH solution and reaction mixtures from light exposure.

   1. Prepare a DPPH solution in 70% methanol.
   2. Adjust the DPPH solution to an absorbance of 1.00 ± 0.02 at 517 nm.
   3. Pipette 50 µL of extract at different concentrations into a microplate well.
   4. Add 250 µL of DPPH solution to each well.
   5. Incubate the reaction mixture in the dark at room temperature for 30 min.
   6. Measure absorbance at 517 nm using a microplate spectrophotometer.
   7. Use methanol as a negative control and ascorbic acid as a positive control.
   8. Construct dose–response curves and calculate IC_{50} values to quantify radical scavenging activity.

6. ABTS cation radical scavenging activity

   NOTE: Perform the ABTS radical scavenging assay according to the method described in the literature, with minor modifications[31]. Prepare the $ABTS^+$ radical solution at least 16 h before use and protect it from light.

   1. Generate the ABTS radical solution by incubating the reaction mixture in the dark at room temperature for 16 h.
   2. Dilute 1 mL of ABTS solution with 80 mL of ethanol prior to analysis.
   3. Adjust the absorbance of the diluted ABTS solution to 0.80 ± 0.02 at 734 nm.
   4. Pipette 50 µL of extract at different concentrations into a microplate well.
   5. Add 250 µL of ABTS solution to each well.
   6. Incubate the mixture at room temperature for 10 min.
   7. Measure absorbance at 734 nm using a microplate spectrophotometer.
   8. Construct dose–response curves and calculate IC_{50} values.

7. Ferric reducing antioxidant power (FRAP) assay

   NOTE: Perform the FRAP assay according to the method proposed in the literature, with minor modifications[32]. Prepare the FRAP reagent freshly before use and protect the reaction mixture from light.

   1. Prepare the FRAP reagent according to the referenced protocol.
   2. Pipette 20 µL of extract at different concentrations into a microplate well.
   3. Add 280 µL of freshly prepared FRAP reagent.
   4. Incubate the reaction mixture at room temperature in the dark for 45 min.

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5. Measure absorbance at 593 nm using a microplate spectrophotometer.
6. Generate a calibration curve using Trolox as a standard.
7. Express antioxidant capacity as mg Trolox equivalents (TE) per gram of extract.

4. Total phenolic content (TPC) analysis

NOTE: Determine the total phenolic content (TPC) of *T. claveryi* extracts using the Folin–Ciocalteu method as described in the literature, with minor modifications[33]. Protect the reaction mixtures from light during incubation. Prepare the sodium carbonate solution freshly before use.

1. Prepare extract solutions at a concentration of 1 mg/mL from freshly prepared stock solutions.
2. Pipette 25 µL of extract into a microplate well, add 75 µL of distilled water, and then add 25 µL of Folin–Ciocalteu reagent.
3. Incubate the mixture at room temperature for 6 min.
4. Add 100 µL of 7% (w/v) sodium carbonate solution to each well.
5. Incubate the reaction mixture at room temperature in the dark for 90 min.
6. Measure absorbance at 665 nm using a microplate spectrophotometer.
7. Prepare a calibration curve using gallic acid as a standard and calculate total phenolic content, expressing the results as mg gallic acid equivalents (GAE) per mL of extract.

5. Phytochemical analysis using LC-MS/MS

NOTE: Perform LC-MS/MS analysis according to previously published chromatographic methods with minor modifications[34]. Filter all samples and mobile phases through 0.22 µm membrane filters and degas the mobile phases prior to analysis.

1. Select the extract exhibiting the highest total phenolic content for quantitative phytochemical profiling.
2. Perform chromatographic separation using a reversed-phase C18 column maintained at 30°C.
3. Prepare the mobile phase consisting of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile, set the flow rate to 0.4 mL/min, adjust the injection volume to 5 µL, and set the total run time to 17 min.
4. Conduct mass spectrometric detection under electrospray ionization (ESI) conditions using nitrogen as the drying, sheath, and nebulizing gas.
5. Set the drying gas temperature to 350 °C with a flow rate of 12 L/min, set the sheath gas temperature to 250 °C with a flow rate of 5 L/min, and adjust the nebulizer pressure to 55 psi.
6. Prepare external calibration curves for each phenolic standard and quantify the analytes using the corresponding calibration curves.

6. Antimicrobial activity test

NOTE: Evaluate antibacterial activity using the agar well diffusion method according to established microbiological protocols. Perform all microbiological procedures under aseptic conditions in a biosafety cabinet. Sterilize all materials before and after use.

1. Select the following type strains for antimicrobial testing: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 14579, *Staphylococcus epidermidis* ATCC 12228, *Listeria monocytogenes* ATCC 19115, *Acinetobacter baumannii* ATCC 17978, *Shigella dysenteriae* ATCC 13313, *Klebsiella pneumoniae* ATCC 13883, and *Enterobacter aerogenes* ATCC 13048.
2. Inoculate each bacterial strain into tryptone soy broth (TSB) and incubate at the optimal growth temperature until reaching an optical density of OD_{600} = 0.7.
3. Spread the bacterial cultures uniformly onto tryptone soy agar (TSA) plates using a sterile swab.
4. Punch wells aseptically into the agar using a sterile cork borer.
5. Add 100 µL of each extract (100 µg/mL) into the corresponding wells.
6. Incubate the plates at 37 °C for 18 h.
7. Measure the diameters of the inhibition zones surrounding each well to assess antimicrobial activity.
8. Perform all experiments in triplicate and express the results as mean values.

7. Biofilm inhibition

NOTE: Evaluate anti-biofilm activity using a crystal violet staining assay against reference bacterial strains. Perform all procedures under aseptic conditions and avoid disturbing adhered biofilms during washing steps.

1. Select *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 as reference strains and culture them in TSB at 37 °C until reaching the logarithmic growth phase.
2. Adjust the bacterial suspensions to an optical density of OD_{600} = 0.132.

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3. Pipette 100 µL of extract solutions (0.625–10 mg/mL) into sterile 96-well polystyrene microtiter plates, add 100 µL of bacterial suspension to each well, and mix gently.
4. Incubate the plates at 37 °C for 24 h. Use wells containing TSB alone as blanks and wells containing bacterial suspension without extract as controls.
5. Remove non-adherent cells carefully, wash the wells twice with distilled water, and allow the plates to air-dry.
6. Add 200 µL of 0.4% (w/v) crystal violet solution to each well and incubate for 30 min.
7. Remove excess stain, rinse the wells twice with distilled water, and allow the plates to dry completely.
8. Add ethanol to solubilize the bound dye and measure absorbance at 595 nm using a microplate reader.
9. Calculate antibiofilm activity using Equation (2).

$$\text{Antibiofilm activity (\%)} = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \text{x } 100 \quad (2)$$

8. Cell culture and cytotoxic activity analysis (MTT assay)

NOTE: Evaluate cytotoxic effects using the MTT assay according to standard cell viability protocols. Perform all procedures under sterile conditions in a biosafety cabinet and pre-warm media and reagents to 37 °C before use.

1. Obtain the human neuroblastoma cell line SH-SY5Y from an accredited cell repository and culture the cells in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine in 25 cm^2 culture flasks.
2. Maintain the cultures at 37 °C in a humidified atmosphere containing 5% CO_2 and grow the cells to approximately 80% confluence.
3. Detach the cells by trypsinization, seed them into 96-well plates at a density of 2 × 10^4 cells per well, and incubate for 24 h to allow attachment.
4. Treat the cells with *T. claveryi* extracts at concentrations of 150, 300, 600, 1200, and 2000 µg/mL and incubate for 24 h.
5. Remove the culture medium, add 100 µL of fresh medium containing 10 µL of MTT solution to each well, and incubate for 4 h at 37 °C.
6. Aspirate the medium, dissolve the formazan crystals by adding 100 µL of dimethyl sulfoxide (DMSO) to each well, and measure absorbance at 570 nm using a microplate spectrophotometer.
7. Perform all experiments in triplicate.

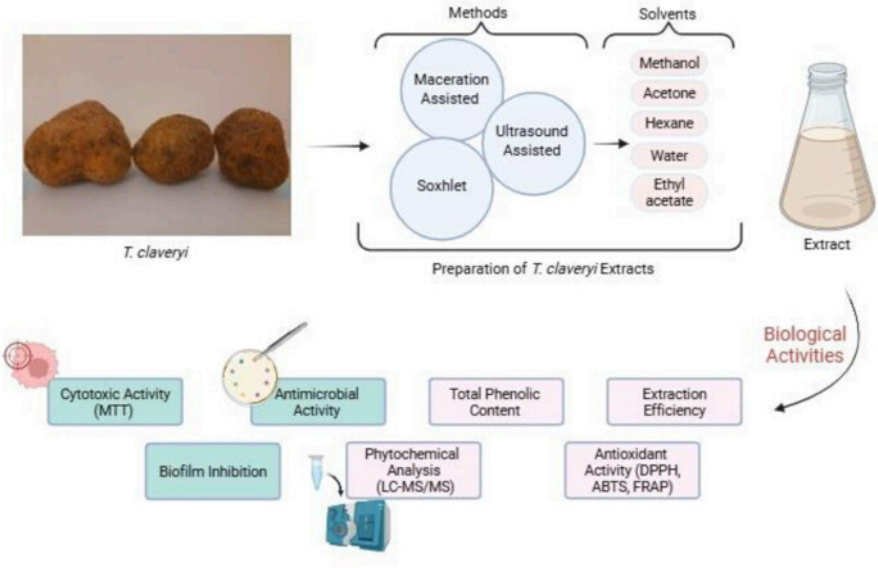

Figure 1: Schematic overview of the experimental workflow. Please click here to view a larger version of this figure.

9. Statistical analysis

1. Perform statistical analyses using appropriate statistical software packages and compare control and treatment groups using Student's *t*-test or two-way analysis of variance (ANOVA), as appropriate.
2. Express all data as mean ± standard error of the mean (SEM).

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3. Define statistical significance at $p < 0.05$.
4. Indicate statistical significance in figures using asterisks as follows: **** for $p < 0.0001$, *** for $p < 0.001$, ** for $p < 0.01$, and * for $p < 0.05$, and use "ns" to denote non-significance.

Results

Molecular identification of *T. claveryi*

PCR amplification of the ITS region was performed using the universal primers ITS1 and ITS4 for molecular identification of *T. claveryi*. The resulting sequences were analyzed and compared against reference sequences in the NCBI database using the BLAST algorithm, allowing definitive species identification (**Supplementary file 1**).

Extraction efficiency

Extraction efficiencies varied depending on the extraction method and solvent polarity (**Table 1**). For the MAE method, extraction efficiency decreased in the following order: water > methanol > ethyl acetate > acetone > n-hexane (2.15–15.01%). The SE and UAE methods showed similar solvent-dependent trends, with extraction efficiency decreasing in the following order: water > methanol > ethyl acetate > n-hexane > acetone (SE: 2.58–9.53%; UAE: 3.01–18.40%). The highest extraction efficiency (18.40%) was obtained with the aqueous extract produced by the UAE method, whereas the lowest (2.15%) was observed with the n-hexane extract produced by the MAE method. This comparison primarily highlights differences in extraction efficiency among extraction methods within the same solvent system and across solvent polarities.

Table 1: Extraction efficiency (%) of *T. claveryi* extracts obtained using different extraction methods and solvents. This table summarizes the extraction yields obtained using maceration, Soxhlet, and ultrasonic-assisted extraction with different solvents. Abbreviations; MAE = maceration assisted extraction; SE = Soxhlet extraction; UAE = ultrasound assisted extraction. Please click here to download this Table.

Antioxidant activity

According to the DPPH assay results (**Figure 2A**), the aqueous extract obtained by the MAE method exhibited the highest radical scavenging activity with an IC_{50} value of 12.03 µg/mL compared to other extraction methods and solvents ($p < 0.05–0.001$). Aqueous extracts obtained by UAE and SE methods showed IC_{50} values of 15.82 µg/mL and 17.87 µg/mL, respectively, and there was no statistically significant difference between these two extracts ($p > 0.05$). It was determined that the SE method had the lowest DPPH radical scavenging capacity among the solvents, and n-hexane extract had the highest IC_{50} (325.69 µg/mL) ($p < 0.0001$). According to the ABTS assay, no significant difference was observed between MAE and UAE aqueous extracts (IC_{50} = 9.83 µg/mL; $p > 0.05$). However, MAE aqueous extract exhibited significantly higher antioxidant activity than SE aqueous extract (IC_{50} = 15.67 µg/mL; $p < 0.05$). Consistent with the DPPH results, the lowest ABTS radical scavenging activity was detected in the n-hexane extract obtained by the SE method (IC_{50} = 338.37 µg/mL; $p < 0.0001$) (**Figure 2B**). FRAP analysis revealed that aqueous extracts obtained by all three extraction methods exhibited the highest reducing power, with values of 206.81, 150.65, and 132.82 mg Trolox equivalent/g dry matter for MAE, UAE, and SE, respectively. Comparison of aqueous extracts showed significant differences between all extraction methods ($p < 0.0001$). The lowest FRAP values were observed for MAE ethyl acetate extract and SE acetone extract, with values of 59.05 and 60.90 mg Trolox equivalents/g dry matter, respectively ($p < 0.0001$) (**Figure 2C**).

Total phenolic content (TPC)

Total phenolic content varies depending on the extraction method and solvent polarity (**Figure 2D**). For the MAE and SE methods, total phenolic content decreased in the following order: water > methanol > ethyl acetate > acetone > n-hexane (MAE: 24.35–65.87 mg GAE/mL; SE: 13.26–53.32 mg GAE/mL). For the UAE method, a similar trend was observed, with total phenolic content decreasing as follows: water > methanol > ethyl acetate > n-hexane > acetone (23.27–58.82 mg GAE/mL). Statistical analysis indicated that the aqueous extracts obtained by all extraction methods were significantly different from each other ($p < 0.01–0.001$). The highest phenolic content was observed in the aqueous extract obtained by MAE (65.87 mg GAE/mL). In contrast, the n-hexane extract obtained by the SE method exhibited a significantly lower phenolic content compared to the MAE method (13.26 mg GAE/mL ; $p < 0.01$) and represented the lowest value among all extracts.

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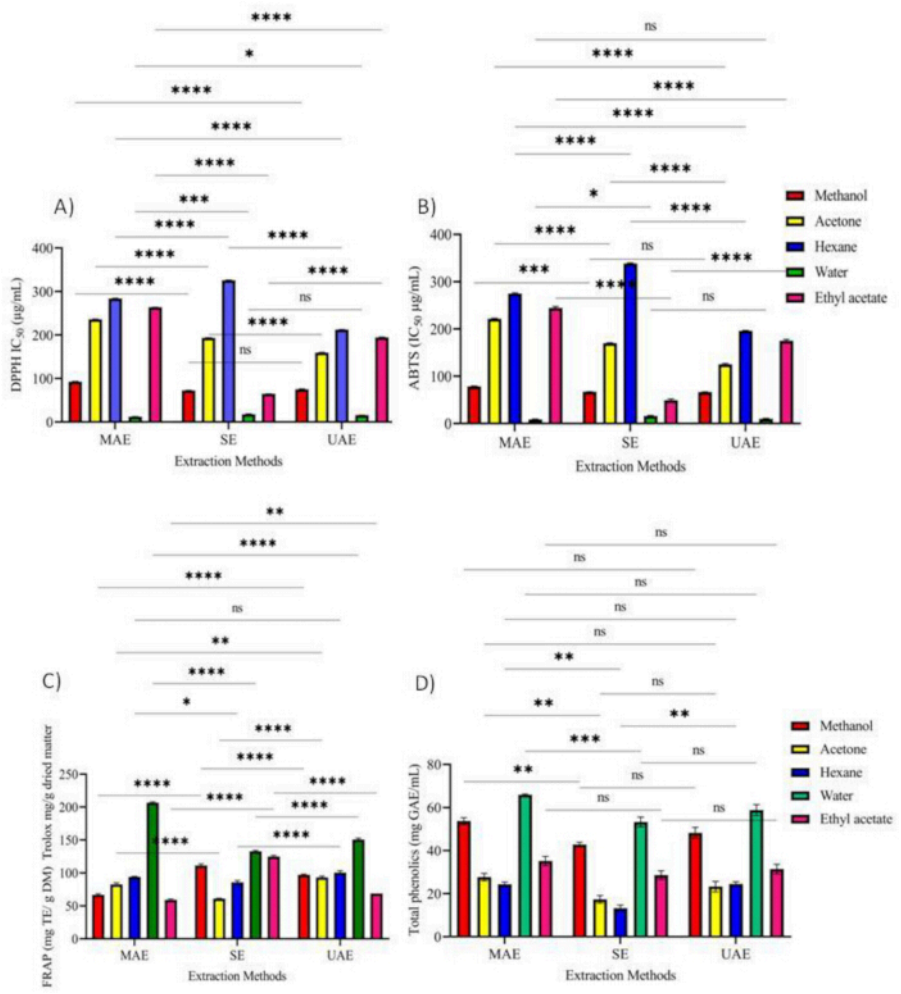

Figure 2: Antioxidant activities and total phenolic results of *T. claveryi* extracts prepared using different extraction methods and five different solvents. Data were obtained from three independent experiments (n = 3) and are expressed as mean ± SD. For DPPH and ABTS assays, results are presented as IC_{50} values. Please click here to view a larger version of this figure.

Polyphenolic analysis with LC-MS/MS

In this study, a total of 35 phytochemical metabolites were used as reference standards for LC-MS/MS analysis. Polyphenolic compounds present in the MAE aqueous extract, which exhibited the highest total phenolic content, were identified based on their MS spectra and retention times compared with reference standards (**Supplementary file 2**). According to the LC-MS/MS analysis, nine phenolic compounds were identified in the MAE water extract, including quinic acid, fumaric acid, keracyanin chloride, cyanidin-3-O-glucoside, chlorogenic acid, peonidin-3-O-glucoside, rosmarinic acid, vanillin, and quercetin (**Table 2**). Quantification of the identified compounds was performed using calibration curves generated from seven different concentrations for each analyte. When ranked from highest to lowest concentration, the detected compounds were quinic acid (2800.37 µg/L), chlorogenic acid (358.85 µg/L), fumaric acid (243.87 µg/L), vanillin (46.80 µg/L), quercetin (42.38 µg/L), cyanidin-3-O-glucoside (27.23 µg/L), rosmarinic acid (13.60 µg/L), peonidin-3-O-glucoside (11.60 µg/L), and keracyanin chloride (8.64 µg/L) (**Table 2**).

Table 2: Quantitative LC-MS/MS analysis of phenolic compounds in the aqueous extract of *T. claveryi* obtained by maceration-assisted extraction. This table presents the identification and quantification of major phenolic compounds detected in the aqueous extract. Abbreviations; ND = not detected; RT = retention time. Please click here to download this Table.

Antimicrobial activity

The antimicrobial activity of *T. claveryi* extracts prepared using three different extraction methods (MAE, SE, and UAE) and five different solvents (methanol, acetone, n-hexane, water, and ethyl acetate) was investigated by the well diffusion method. Although different extraction methods were applied, all extracts exhibited comparable antimicrobial activity against the tested pathogens.

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No marked differences were observed between MAE, SE, and UAE methods; however, extracts obtained by the UAE method generally produced slightly larger inhibition zones. The type of solvent did not result in a statistically significant overall difference in antimicrobial activity, although solvent-dependent variations were observed depending on the bacterial strain. The inhibition zone diameters of the extracts against the tested strains are presented in **Table 3**.

Table 3: Inhibition zone diameters of *T. claveryi* extracts obtained using three different extraction methods and five different solvents against pathogenic microorganisms. This table shows the antimicrobial activity of different extracts expressed as inhibition zone diameters against selected pathogenic strains. Abbreviations; M = Maceration, S = Soxhlet; U = Ultrasonic; 1 = Methanol; 2 = Acetone; 3 = Hexane; 4 = Water; 5 = Ethyl Acetate. Please click here to download this Table.

Overall, *T. claveryi* extracts demonstrated notable antimicrobial activity against all clinically relevant type strains tested. Particularly strong inhibitory effects were observed against *Bacillus cereus* ATCC 14579 and *Acinetobacter baumannii* ATCC 17978, which are associated with foodborne infections and multidrug-resistant hospital-acquired infections, respectively. Representative images of inhibition zones obtained using different extraction methods and solvents are shown in **Figure 3**.

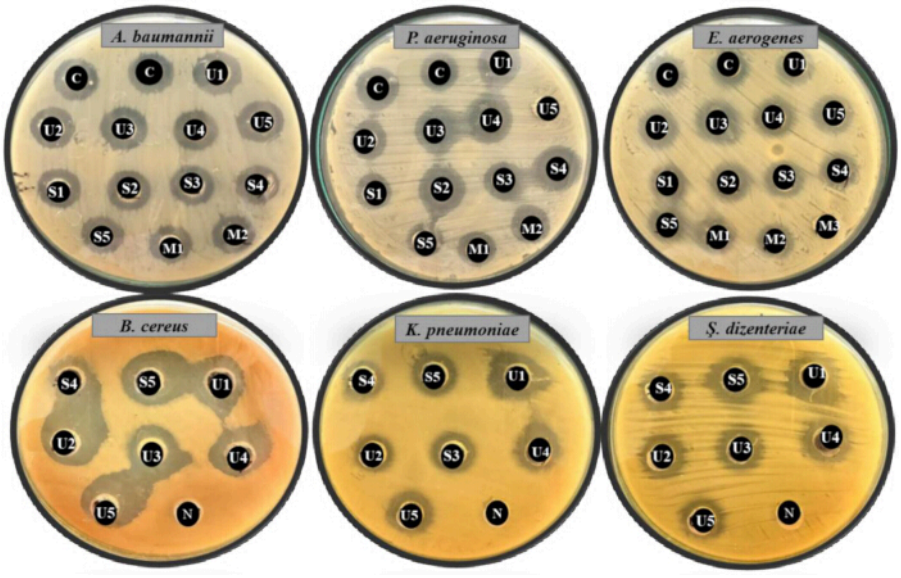

Figure 3: Representative images of inhibition zone diameters produced by *T. claveryi* extracts against selected pathogenic microorganisms. Abbreviations; M(MAE) = maceration-assisted extraction; S = Soxhlet extraction; U(UAE) = ultrasound-assisted extraction; 1, methanol; 2, acetone; 3, n-hexane; 4, water; 5, ethyl acetate; C, positive control; N, negative control. Please click here to view a larger version of this figure.

Anti-biofilm Inhibition Assay

The antibiofilm activity of T. claveryi extracts prepared using three extraction methods and five solvents was evaluated against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 type strains. The extracts obtained using different extraction methods and solvents exhibited concentration-dependent anti-biofilm activity against both strong biofilm-producing strains. Based on the obtained data, no statistically significant differences were observed in overall anti-biofilm activity among the extraction methods or solvents. However, when the methods were compared, the MAE method exhibited relatively higher anti-biofilm efficacy than the other extraction methods, and among the solvents, n-hexane showed slightly higher activity. The highest biofilm inhibition was observed against E. coli ATCC 25922 at the highest concentration tested (10 µg/mL), with an inhibition rate of 64.2% using the MAE method and n-hexane solvent (**Figure 4**). For P. aeruginosa ATCC 27853, the maximum biofilm inhibition was determined as 57.3% under the same extraction conditions (**Figure 5**). Overall, *T. claveryi* extracts demonstrated higher anti-biofilm activity against *E. coli* compared to *P. aeruginosa*.

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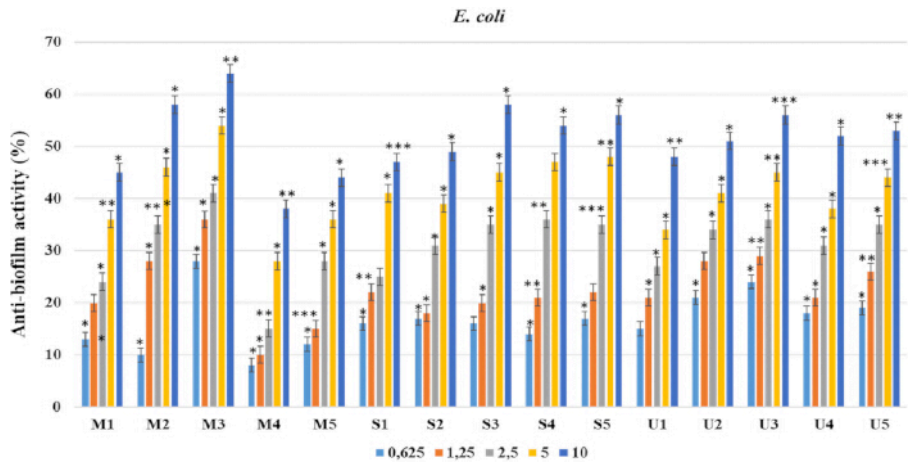

Figure 4: Anti-biofilm activity of *T. claveryi* phytochemicals prepared using three different extraction methods and five different solvents on *E. coli* ATCC 25922 strains. Abbreviations; M(MAE) = maceration-assisted extraction; S = Soxhlet extraction; U(UAE) = ultrasound-assisted extraction; 1, methanol; 2, acetone; 3, n-hexane; 4, water; 5, ethyl acetate. Data were obtained from three independent experiments (n = 3). Please click here to view a larger version of this figure.

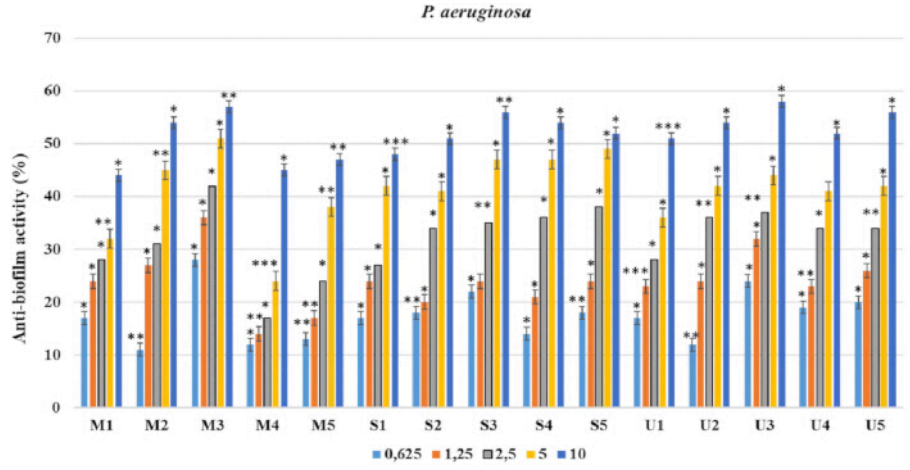

Figure 5: Anti-biofilm activity of *T.claveryi* phytochemicals prepared using three different extraction methods and five different solvents on *P. aeruginosa* ATCC 25922 strain. Abbreviations; M(MAE) = maceration-assisted extraction; S = Soxhlet extraction; U(UAE) = ultrasound-assisted extraction; 1, methanol; 2, acetone; 3, n-hexane; 4, water; 5, ethyl acetate; . Data were obtained from three independent experiments (n = 3). Please click here to view a larger version of this figure.

Cytotoxic activity

The cytotoxic activities of *T. claveryi* extracts prepared using three extraction methods (MAE, SE, and UAE) and five solvents (methanol, acetone, hexane, water, and ethyl acetate) on the human neuroblastoma cell line SH-SY5Y are shown in **Figures 6–8**. For the MAE method, the methanol extract (MAE/1) induced a significant decrease in cell viability at 150, 300, and 2000 µg/mL compared to the control group ($p < 0.05 – p < 0.001$) (**Figure 6**). In contrast, the acetone (MAE/2), hexane (MAE/3), and water (MAE/4) extracts did not significantly affect viability at 150 and 300 µg/mL. However, the ethyl acetate extract (MAE/5) caused a significant reduction in cell viability at all tested doses, showing a pronounced dose-dependent effect ($IC_{50(MAE/5)}$ = 1304.83 ± 102.64 µg/mL). A similar dose-dependent decrease was also observed for the MAE acetone extract (MAE/2) at higher concentrations. Significant solvent-dependent differences within the MAE method emerged at 1200 and 2000 µg/mL ($p < 0.05 – p < 0.0001$). Using the SE method, all solvent extracts significantly reduced SH-SY5Y cell viability at 150 µg/mL compared to the control group ($p < 0.05 – p < 0.0001$) (**Figure 7**). The methanol extract (SE/1) exhibited the strongest dose-dependent cytotoxic effect. The water extract (SE/4) showed a comparatively weaker dose-dependent response, whereas acetone (SE/2) and hexane (SE/3) extracts displayed marked reductions in viability at intermediate and higher doses. No significant differences between solvents

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were detected at 150 µg/mL ($p > 0.05$); however, at 600 µg/mL, the ethyl acetate extract (SE/5) showed significantly higher cytotoxicity than the other solvent groups ($IC_{50(SE/5)}$ = 394.18 ± 72.08µg/mL). ($p < 0.05$ – $p < 0.0001$).For the UAE method, all solvent extracts reduced SH-SY5Y cell viability at 150 µg/mL, with statistically significant effects observed for all groups except the acetone extract (UAE/2) ($p < 0.001$ –$p < 0.0001$) (**Figure 8**). The ethyl acetate extract (UAE/5) exhibited the lowest viability at this dose. The UAE acetone extract (UAE/2) induced a significant, dose-dependent reduction in viability starting from 300 µg/mL, while the water extract (UAE/4) caused significant decreases in viability at all tested concentrations ($IC_{50(UAE/2}$ = 988.45 ± 38.32µg/mL). Solvent comparisons within the UAE method revealed that at 150 µg/mL, only the UAE/5 group differed significantly from UAE/2 ($p < 0.001$), whereas at the highest dose, the UAE/2 group differed significantly from all other extracts except UAE/5 ($p < 0.0001$)

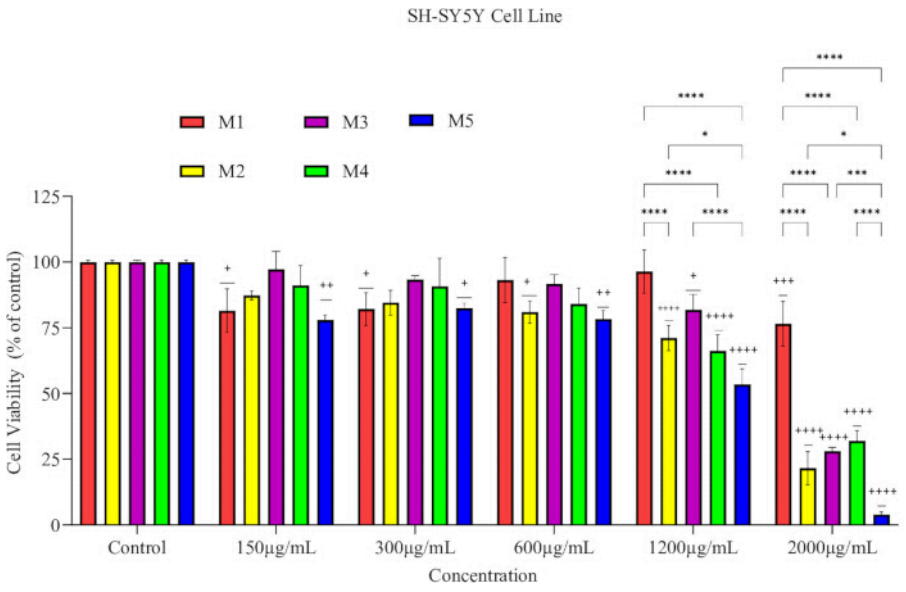

Figure 6: Cytotoxic activity of *T. claveryi* extracts obtained by maceration extraction (M) using five different solvents against human SH-SY5Y neuroblastoma cells. Solvents were methanol (1), acetone (2), n-hexane (3), water (4), and ethyl acetate (5). Data were obtained from three independent experiments (n = 3). ⁺Indicates a statistically significant difference compared to the control group, while * indicates a statistically significant difference between the methods. Please click here to view a larger version of this figure.

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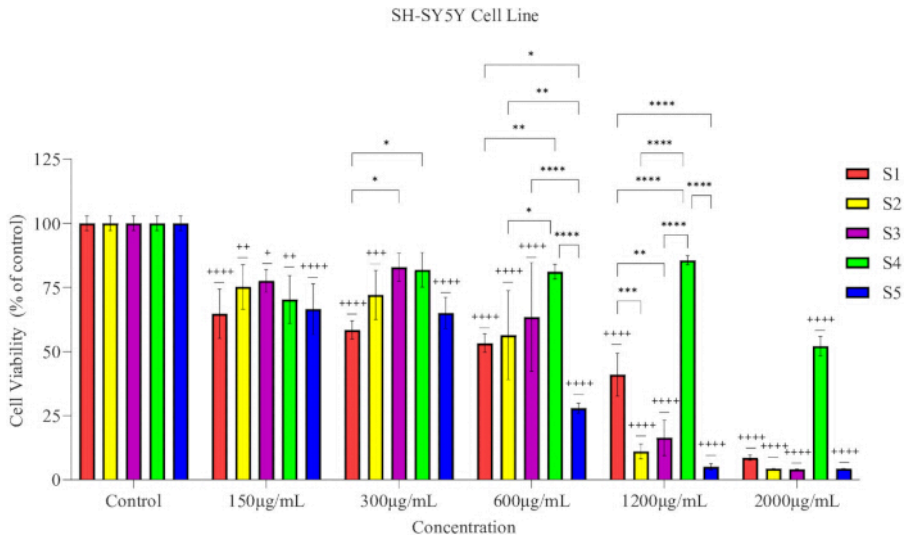

Figure 7: Cytotoxic activity of *T. claveryi* extracts obtained by Soxhlet extraction (S) using five different solvents against human SH-SY5Y neuroblastoma cells. Solvents were methanol (1), acetone (2), n-hexane (3), water (4), and ethyl acetate (5). Data were obtained from three independent experiments (n = 3). ⁺Indicates a statistically significant difference compared to the control group, while * indicates a statistically significant difference between the methods. Please click here to view a larger version of this figure.

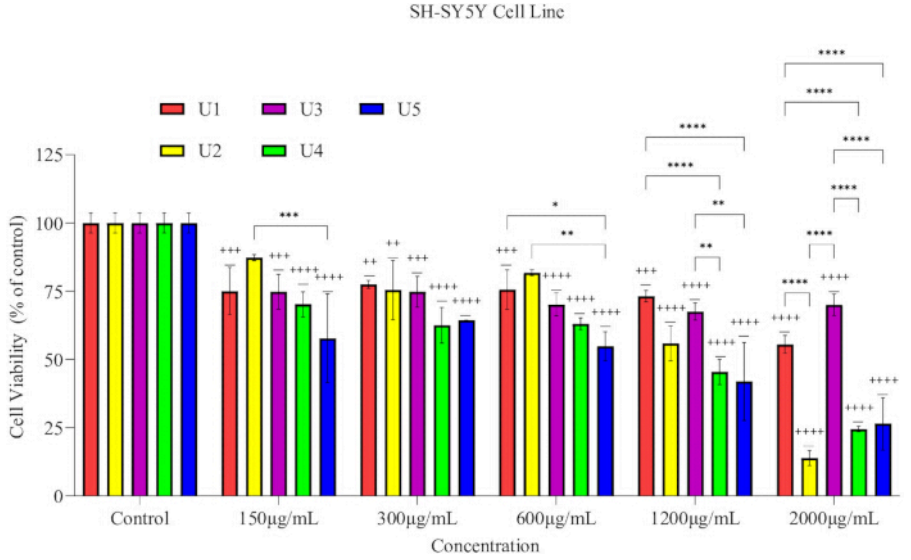

Figure 8: Cytotoxic activity of *T. claveryi* extracts obtained by ultrasound-assisted extraction (U) using five different solvents against human SH-SY5Y neuroblastoma cells. Solvents were methanol (1), acetone (2), n-hexane (3), water (4), and ethyl acetate (5). Data were obtained from three independent experiments (n = 3). ⁺Indicates a statistically significant difference compared to the control group, while * indicates a statistically significant difference between the methods. Please click here to view a larger version of this figure.

This study demonstrated that both the extraction method and solvent polarity significantly influenced the extraction efficiency and *in vitro* bioactivities of *T. claveryi* extracts. Among the tested methods, UAE generally yielded higher extraction efficiencies, whereas MAE aqueous extracts exhibited the strongest antioxidant capacity. Antimicrobial and antibiofilm activities showed comparatively limited variation across extraction strategies, whereas cytotoxic effects on SH-SY5Y cells were dose-dependent and varied with solvent–method combinations. These findings highlight distinct method-dependent trends across biological endpoints.

Supplementary Table 1: Species identification results report. This table presents the results of species identification analysis performed for *T. claveryi* samples. Please click here to download this file.

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Supplementary Table 2: LC-MS/MS chromatograms and quantitative data for phenolic compounds identified in the aqueous extract of *T. claveryi*. This table presents the LC-MS/MS chromatograms and quantitative analysis of phenolic compounds detected in the aqueous extract. Please click here to download this file.

Discussion

The present study establishes a comparative and standardized extraction–bioactivity platform for *T. claveryi*, demonstrating that the extraction strategy is a decisive determinant of both chemical composition and biological functionality. The primary methodological strength of this protocol lies in the strict control of extraction parameters, particularly the fixed solid–liquid ratio, standardized solvent volume, identical post-extraction processing, and uniform stock solution preparation prior to bioassays. These steps represent critical control points within the workflow. Variability in solvent volume, incomplete solvent removal, or inconsistent reconstitution concentrations can significantly bias antioxidant or cytotoxic comparisons. Therefore, maintaining identical downstream preparation conditions ensured that observed biological differences reflected qualitative compositional changes rather than concentration artifacts. Additionally, temperature regulation during UAE and aqueous SE was essential. Cavitation during ultrasound extraction generates localized high-energy microenvironments that may enhance mass transfer but can also destabilize sensitive metabolites if not carefully controlled. Intermittent cooling in this study served as a practical troubleshooting step to maintain phenolic stability.

Extraction yield results demonstrated a clear polarity-dependent trend, with water producing the highest yield and n-hexane the lowest. This pattern is consistent with established solvent polarity indices and previous reports indicating preferential extraction of hydrophilic compounds by high-polarity solvents[35,36,37,38,39]. The enhanced yield observed under UAE conditions aligns with cavitation-mediated disruption of fungal cell walls and increased solvent penetration[40]. However, yield did not directly translate into maximal biological activity. Although the UAE produced the highest extraction yield, the MAE/water extract demonstrated superior antioxidant capacity and the highest total phenolic content. This discrepancy highlights an important methodological insight: extraction efficiency and bioactive enrichment are not synonymous. Prolonged solvent–matrix interaction under controlled, room-temperature maceration may favor selective preservation and gradual diffusion of phenolic acids, whereas more aggressive physical disruption may co-extract non-active matrix components or induce partial structural modification of labile compounds. Therefore, selecting an extraction technique solely based on yield may lead to suboptimal functional performance.

The antioxidant findings showed strong concordance between total phenolic content and DPPH, ABTS, and FRAP responses. Previous investigations of *T. claveryi* and related Terfezia species have reported solvent-dependent variability in antioxidant activity, with methanol, ethanol, or aqueous extracts frequently demonstrating superior radical scavenging properties[27,37,41,42,43,44]. While some discrepancies exist in the literature, these differences likely arise from geographical variation, post-harvest handling, and methodological heterogeneity. In the present study, LC-MS/MS profiling of the MAE/water extract revealed quinic acid and chlorogenic acid as the dominant constituents, along with quercetin, rosmarinic acid, and anthocyanin derivatives. Chlorogenic acid is widely recognized for its multi-mechanistic antioxidant behavior, functioning through both hydrogen atom transfer (HAT) and single electron transfer (SET) pathways[45,46,47,48,49,50]. Its ability to reduce ferric ions in FRAP assays while simultaneously scavenging stable radicals such as DPPH and ABTS provides a mechanistic explanation for the consistent antioxidant superiority of the MAE aqueous extract. Furthermore, the presence of multiple hydroxyl groups in rosmarinic acid enhances radical stabilization through resonance, potentially contributing synergistically to total antioxidant capacity[51]. These findings reinforce the mechanistic link between phenolic enrichment and redox-based assay performance.

Antimicrobial and antibiofilm assays revealed moderate but consistent activity across extraction methods, aligning with previous reports describing solvent-dependent antibacterial properties in Terfezia extracts[52,53,54,55,56,57]. However, these activities were assessed at fixed concentrations, and minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), or minimum biofilm inhibitory concentration (MBIC) values were not determined. Consequently, the antimicrobial findings should be interpreted as comparative screening data rather than quantitative potency assessments. Additionally, the antibiofilm assay relied on crystal violet biomass staining without evaluating biofilm viability or structural integrity, which limits mechanistic interpretation.

Cytotoxic responses exhibited a distinct solvent- and method-dependent pattern. Organic solvent extracts obtained via Soxhlet extraction showed stronger cytotoxic effects on SH-SY5Y neuroblastoma cells, particularly in methanol and acetone fractions. Similar solvent-dependent cytotoxic trends have been reported in truffle species evaluated against various cancer cell lines[42,58] The exhaustive and high-temperature reflux conditions of SE may enhance recovery of lipophilic aglycones, sterols, or other semi-polar secondary metabolites with increased membrane permeability and pro-apoptotic potential. In contrast, aqueous extracts obtained via MAE and UAE, while rich in phenolic antioxidants, may lack sufficient concentrations of lipophilic cytotoxic constituents. This divergence underscores a methodological trade-off between

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maximizing hydrophilic antioxidant recovery and enriching cytotoxic bioactive fractions[59,60]. Cytotoxic evaluation was based solely on the MTT assay, which measures metabolic activity but does not distinguish between apoptosis, necrosis, or cell cycle arrest mechanisms. These limitations represent important considerations for future refinement of the protocol. Another limitation of this study is that LC-MS/MS profiling is restricted to MAE/water extract; therefore, compositional elucidation of more cytotoxic organic fractions remains indirect. Expanded metabolomic characterization across all extract types would strengthen the mechanistic interpretation of the cytotoxic findings.

Despite these constraints, the integrated nature of the present methodological framework represents a significant advancement over studies that focus on a single extraction technique or isolated biological endpoint. By systematically combining extraction yield analysis, total phenolic quantification, multi-assay antioxidant evaluation, antimicrobial and antibiofilm screening, cytotoxic testing, and targeted LC-MS/MS profiling within one standardized workflow, the protocol provides a rational and reproducible platform for extraction-driven bioactivity comparison. This integrated approach facilitates evidence-based selection of extraction conditions depending on the intended functional outcome, whether antioxidant enrichment or cytotoxic potency.

Future applications of this framework may include comprehensive LC-MS/MS profiling of all solvent fractions, bioactivity-guided fractionation to isolate active constituents, incorporation of MIC/MBC/MBIC determinations for quantitative antimicrobial validation, and mechanistic cellular assays investigating apoptosis pathways, oxidative stress modulation, and signaling cascades. Furthermore, process optimization studies integrating response surface methodology could refine solvent–temperature–time parameters for targeted bioactive enrichment. Such extensions would enhance translational relevance and support standardization strategies for developing *T. claveryi*-derived ingredients in functional food, nutraceutical, or complementary therapeutic applications.

Disclosures

The authors declare no competing interests. All data supporting the findings of this study are available within the main manuscript and the supplementary information files. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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