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Article

Microwave-Assisted Synthesis, Lipophilicity and In Vitro Antimicrobial Activity of Hydrazide-Hydrazones of Phenylacetic Acid

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SUPPLEMENTARY MATERIALS

1. Examples of IR, ^{1}H NMR and ^{13}C NMR spectra of synthesized hydrazide-hydrazones of phenylacetic acid

Figure S1. The IR spectrum of compound **3**: *N*′-[(*E*)-(3,5-dibromo-2-hydroxyphenyl)methylidene]-2-phenylacetohydrazide

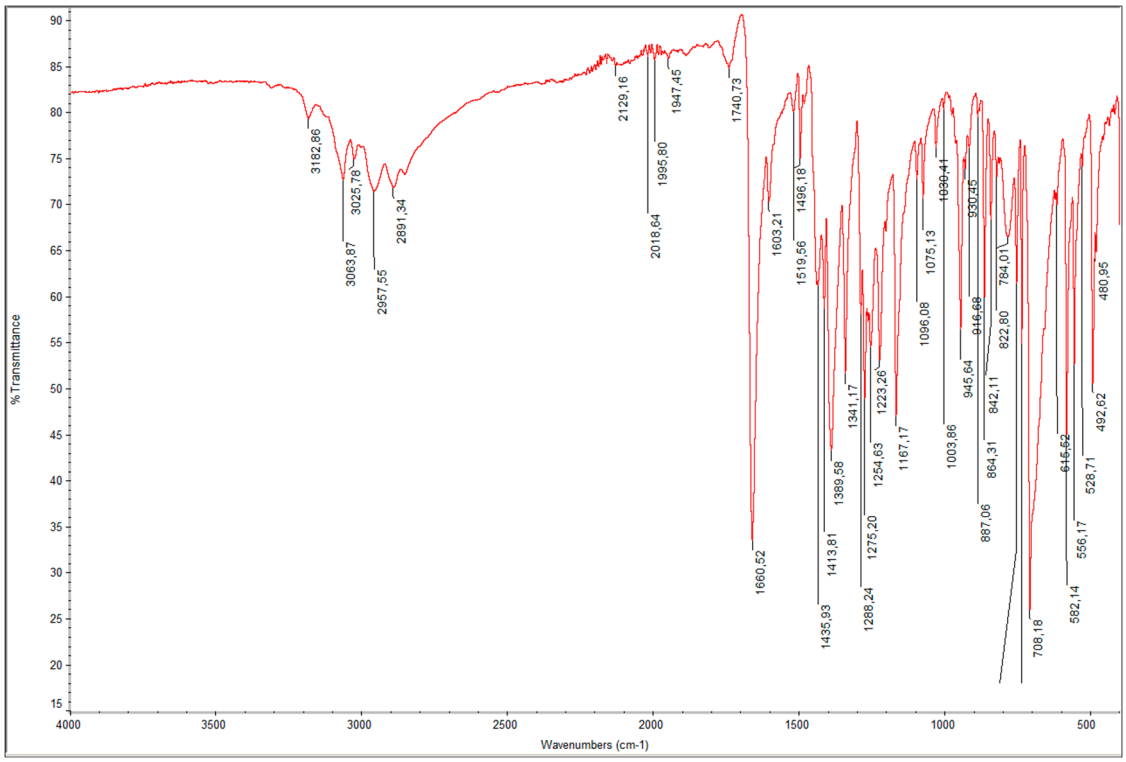


Figure S2. The ^{1}H NMR spectrum of compound **3**: *N*′-[(*E*)-(3,5-dibromo-2-hydroxyphenyl)methylidene]-2-phenylacetohydrazide

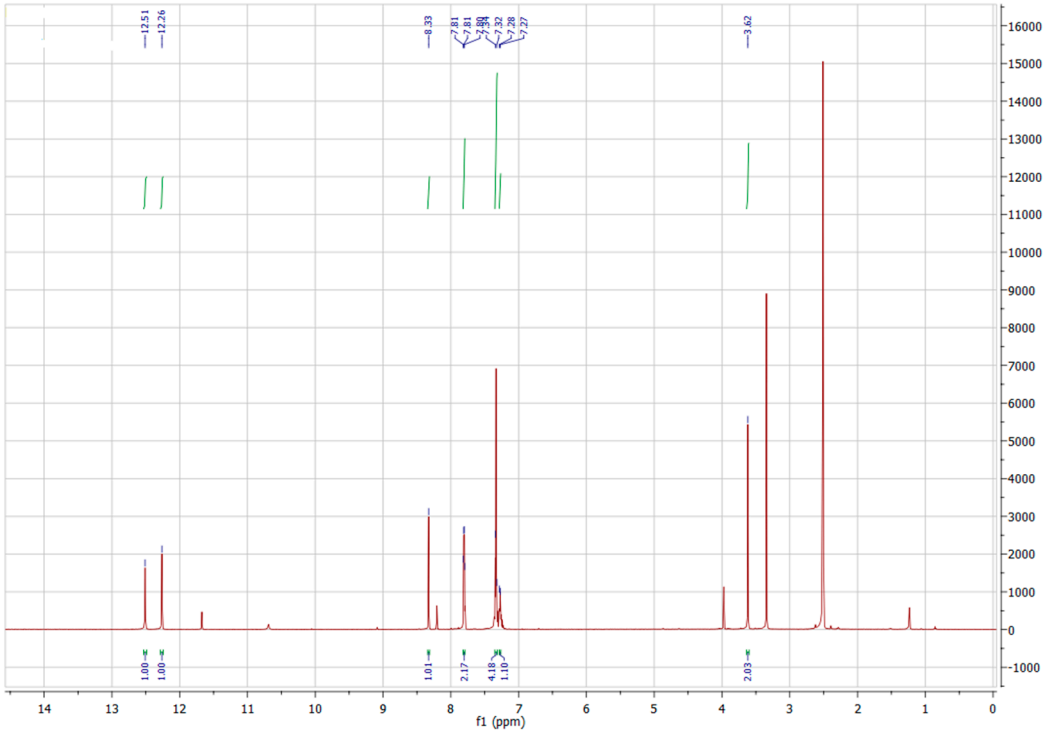


Figure S3. The ^{13}C NMR spectrum of compound **3**: *N′*-[(*E*)-(3,5-dibromo-2-hydroxyphenyl)methylidene]-2-phenylacetohydrazide

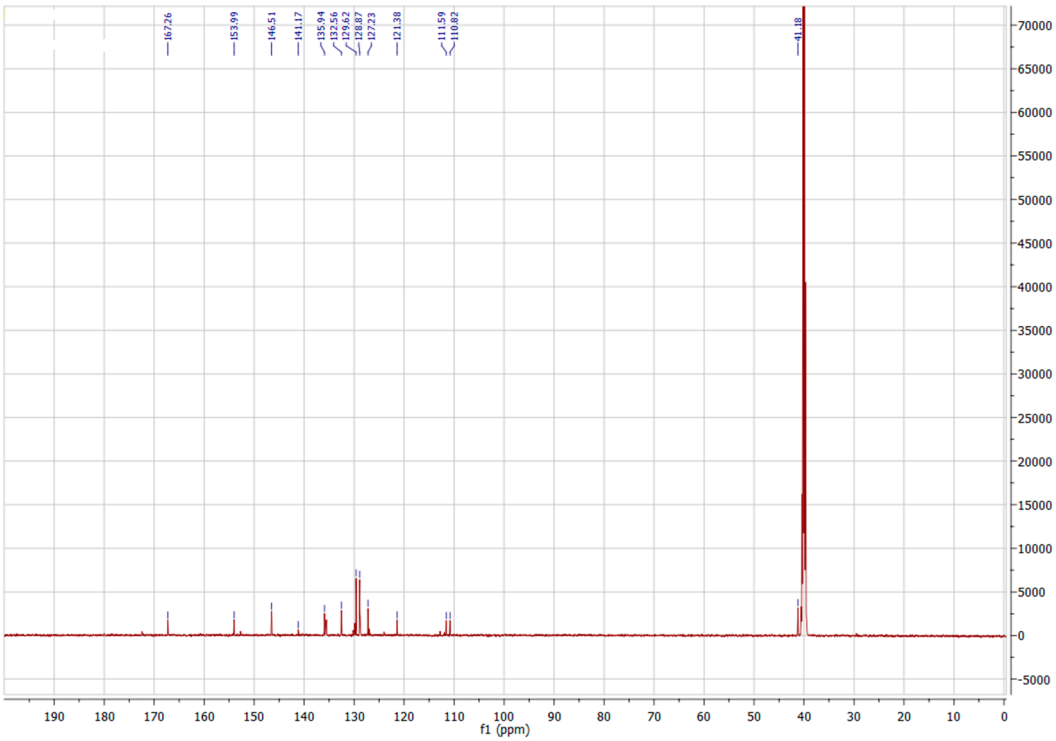


Figure S4. The IR spectrum of compound **7**: *N*′-[(*E*)-(5-chloro-2-hydroxy-3-iodophenyl)methylidene]-2-phenylacetohydrazide

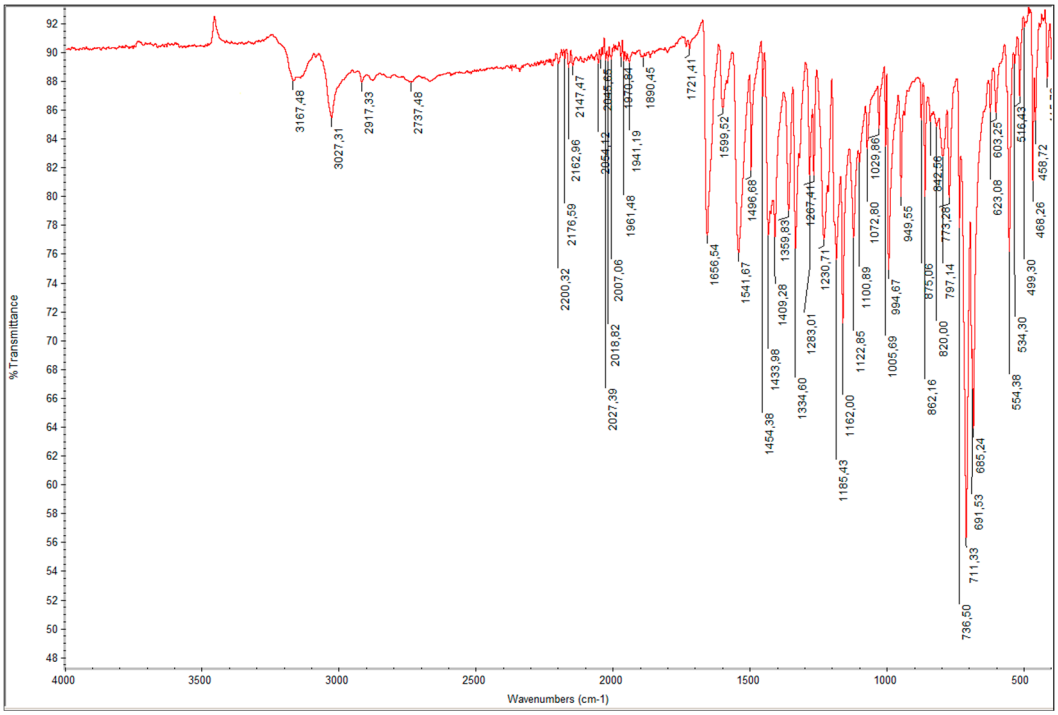


Figure S5. The ^{1}H NMR spectrum of compound **7**: *N*′-[(*E*)-(5-chloro-2-hydroxy-3-iodophenyl)methylidene]-2-phenylacetohydrazide

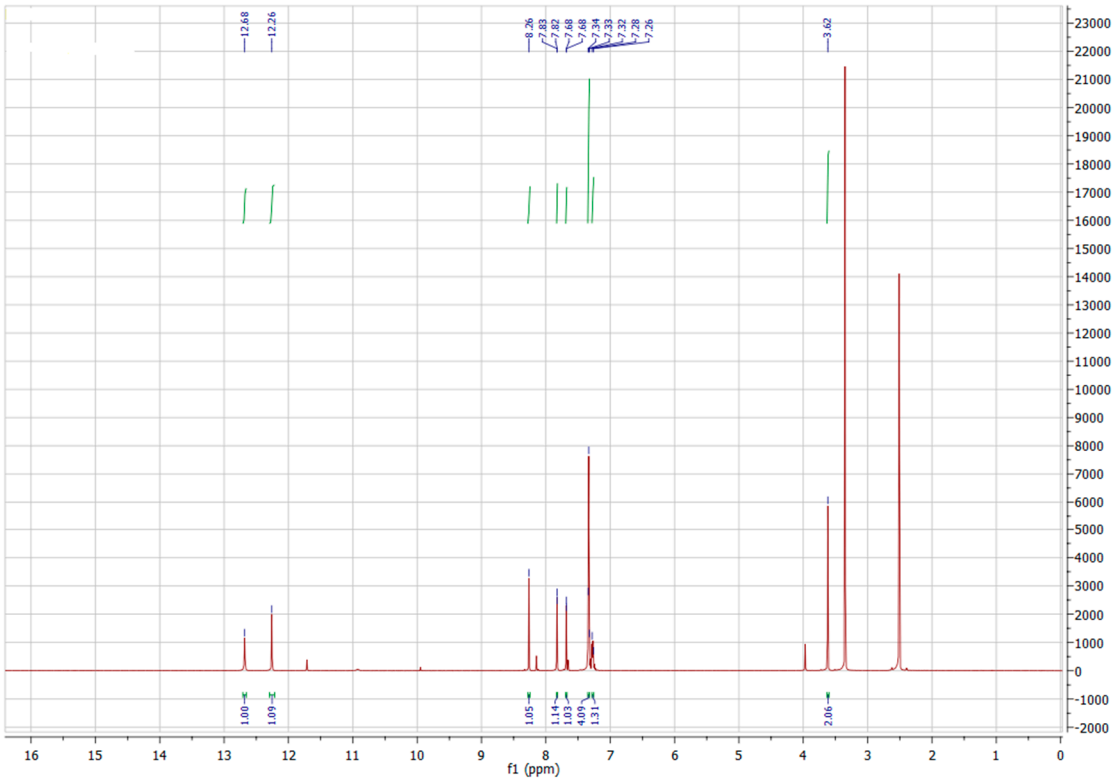


Figure S6. The ^{13}C NMR spectrum of compound **7**: *N*′-[(*E*)-(5-chloro-2-hydroxy-3-iodophenyl)methylidene]-2-phenylacetohydrazide

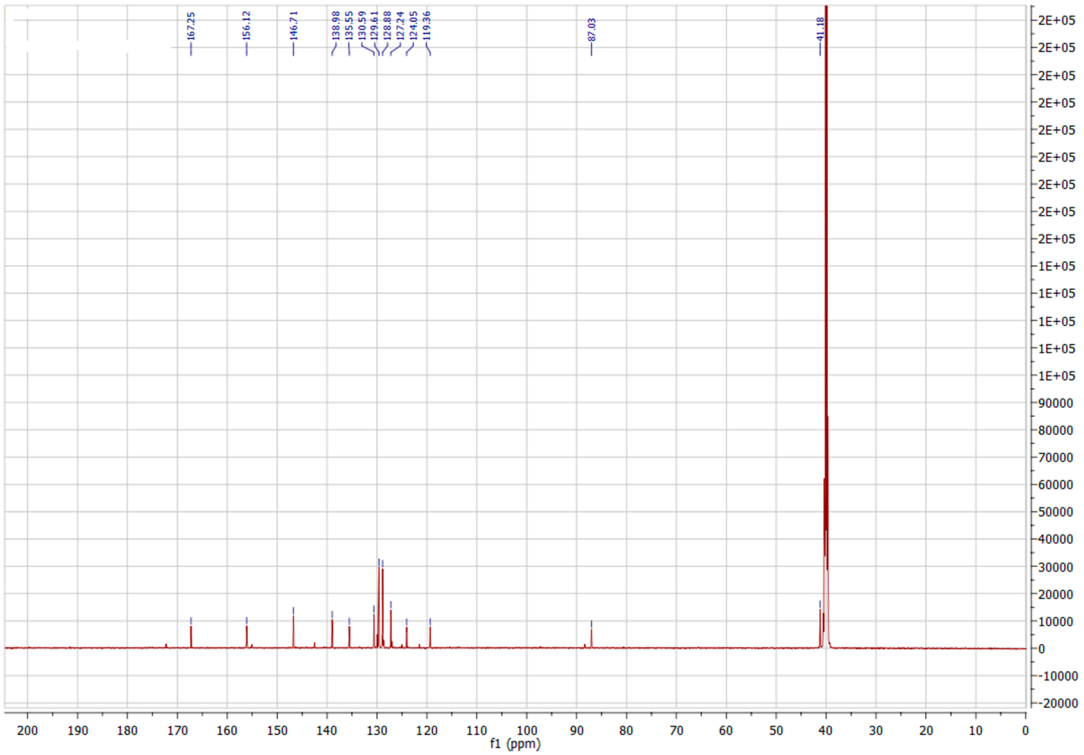


Figure S7. The IR spectrum of compound **8**: *N*′-[(*E*)-(2-hydroxy-3,5-diiodophenyl)methylidene]-2-phenylacetohydrazide

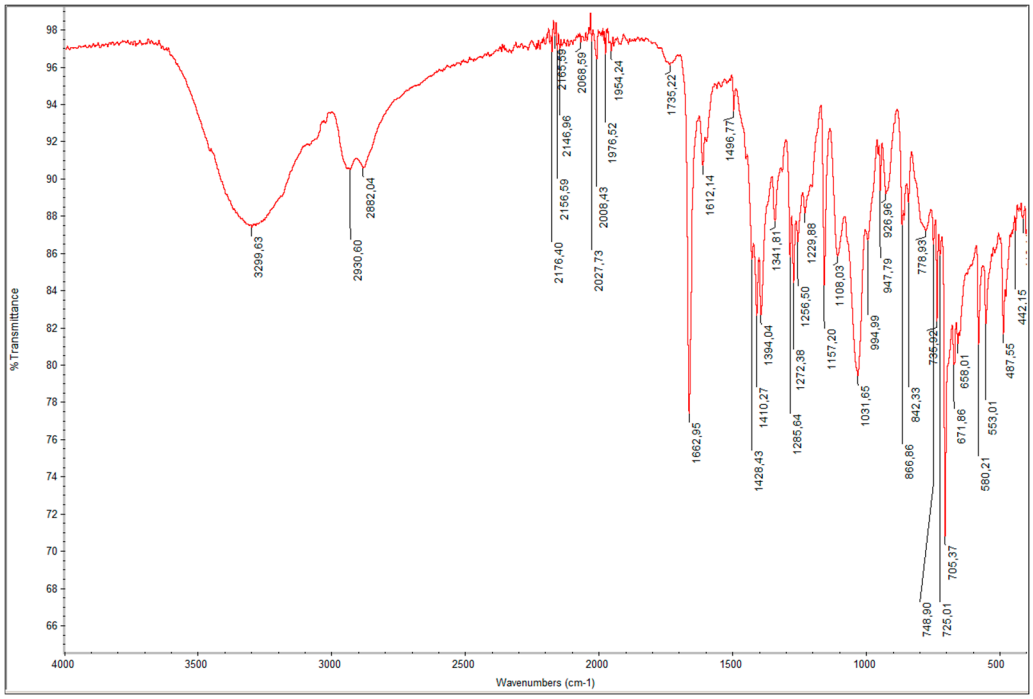


Figure S8. The ^{1}H NMR spectrum of compound **8**: *N*′-[(*E*)-(2-hydroxy-3,5-diiodophenyl)methylidene]-2-phenylacetohydrazide

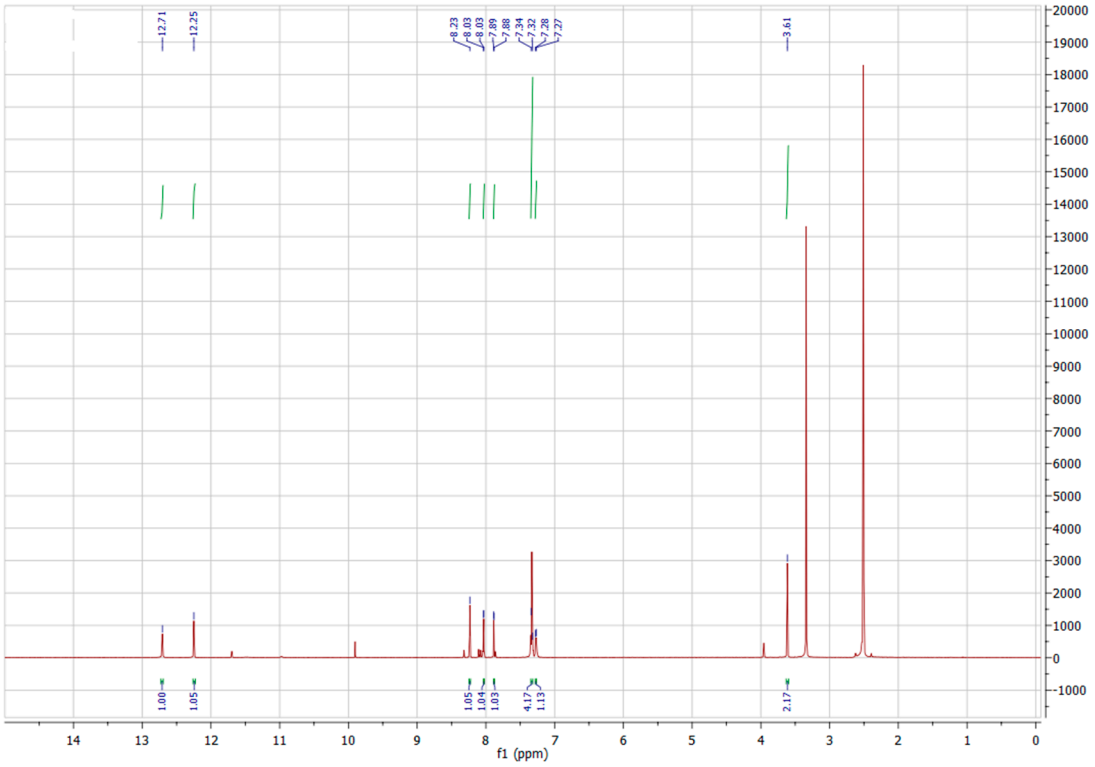
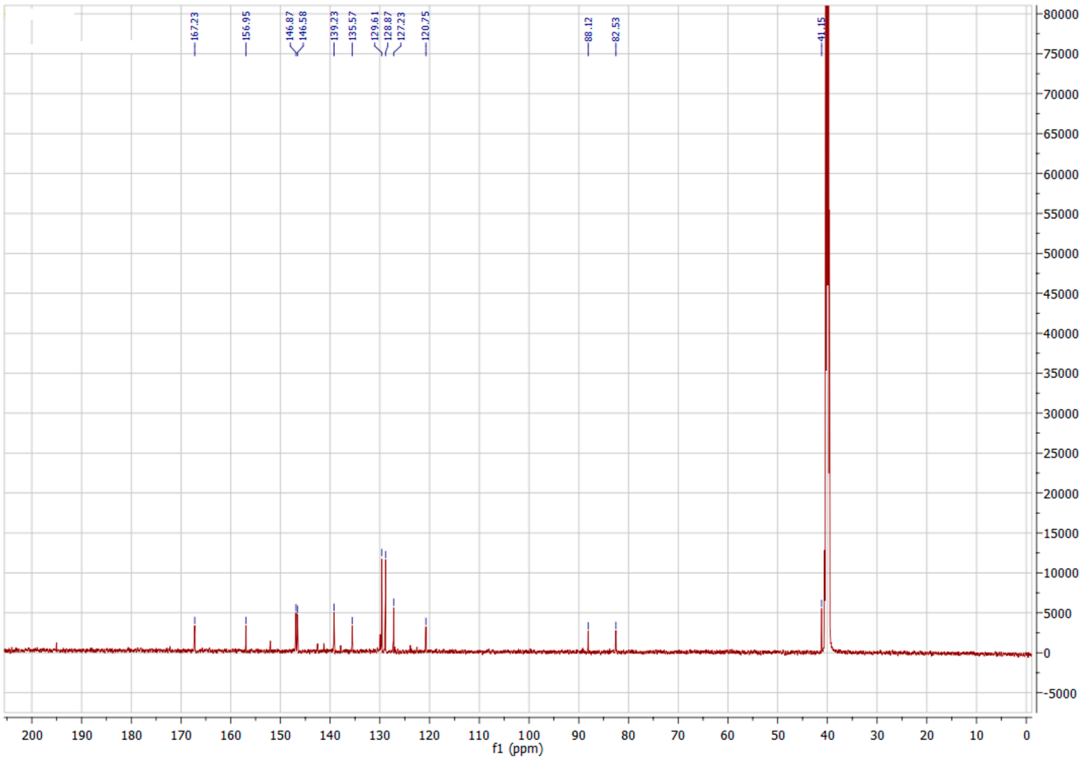


Figure S9. The ^{13}C NMR spectrum of compound **8**: *N*′-[(*E*)-(2-hydroxy-3,5-diiodophenyl)methylidene]-2-phenylacetohydrazide



2. Microbiology - *In vitro* antimicrobial assay – Detailed procedures

The examined compounds **2-16** were screened *in vitro* for antibacterial and antifungal activities using the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) [1] and Clinical and Laboratory Standards Institute guidelines [2] against a panel of reference strains of microorganisms, including Gram-positive bacteria (*Staphylococcus aureus* ATCC 43300 (Methicillin Resistant *S. aureus* – MRSA), *Staphylococcus aureus* ATCC 29213 (Methicillin Susceptible *S. aureus* – MSSA), *Staphylococcus aureus* ATCC 6538 (Methicillin Susceptible *S. aureus* – MSSA), *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 10876), Gram-negative bacteria (*Bordetella bronchiseptica* ATCC 4617, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 12453, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 9027) and fungi belonging to yeasts (*Candida auris* CDC 311903, *Candida albicans* ATCC 10231, *Candida albicans* ATCC 2091, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030 and *Candida krusei* ATCC 14243). The microorganisms came from American Type Culture Collection (ATCC) or Centers for Disease Control (CDC), routinely used for the evaluation of antimicrobials. All the used microbial cultures were first subcultured on nutrient agar or Sabouraud agar at 35°C for 18-24 h or 30°C for 24-48 h for bacteria and fungi, respectively.

The surface of Mueller-Hinton agar (for bacteria) and RPMI 1640 with MOPS (for fungi) were inoculated with the suspensions of bacterial or fungal species. Microbial suspensions were prepared in sterile saline with an optical density of McFarland standard scale 0.5. Samples containing examined compounds were dissolved in sterile water. Subsequently MIC (Minimal Inhibitory Concentration) of the compounds was examined by the microdilution broth method, using their two-fold dilutions in Mueller-Hinton broth (for bacteria) and RPMI 1640 broth with MOPS (for fungi) prepared in 96-well polystyrene plates. Final concentrations of the compounds ranged from 1000 to 0.488 μg/mL. Microbial suspensions were prepared in 0.85% NaCl with an optical density of 0.5 McFarland standard. Next each bacterial or fungal suspension was added per each well containing broth and various concentrations of the examined compounds. After incubation, the MIC was assessed spectrophotometric as the lowest concentration of the samples showing complete bacterial or fungal growth inhibition. Appropriate, growth and sterile controls were carried out. The medium with no tested substances was used also as control. The inhibition of microbial growth was judged by comparison with a control culture prepared without any sample tested. Ciprofloxacin, nitrofurantoin, cefuroxime, ampicillin, vancomycin or nystatin (Sigma-Aldrich Chemicals, St. Louis, MO, USA) were used as a reference antibacterial or antifungal compounds, respectively.

The MBC (Minimal Bactericidal Concentration) or MFC (Minimal Fungicidal Concentration) are defined as the lowest concentration of the compounds that is required to kill a particular bacterial or fungal species. MBC or MFC was determined by removing the culture using for MIC determinations from each well and spotting onto appropriate agar medium. The plates were incubated under appropriate conditions for bacteria and fungi. The lowest compounds concentrations with no visible growth observed was assessed as a bactericidal or fungicidal concentration. All the experiments were repeated three times and representative data are presented [3, 4].

In this study, no bioactivity was defined as a MIC > 1000 μg/mL, mild bioactivity as a MIC in the range 501 – 1000 μg/mL, moderate bioactivity with MIC from 126 to 500 μg/mL, good bioactivity as a MIC in the range 26 – 125 μg/mL, strong bioactivity with MIC between 10 and 25 μg/mL and very strong bioactivity as a MIC < 10 μg/mL [5]. The MBC/MIC or MFC/MIC ratios were calculated in order to determine bactericidal/fungicidal (MBC/MIC ≤ 4, MFC/MIC ≤ 4) or bacteriostatic/fungistatic (MBC/MIC > 4, MFC/MIC > 4) effect of the tested compounds.

References:

1. European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2003) determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. EUCAST discussion document E. Dis 5.1, *Clin. Microbiol. Infect.* **2003**, *9*, 1-7.
2. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. M27-S4. Clinical and Laboratory Standards Institute, Wayne, PA, USA, **2012**.
3. Biernasiuk A.; Kawczyńska M.; Berecka-Rycerz A.; Rosada B.; Gumieniczek A.; Malm A.; Dzitko K.; Łączkowski K.Z.: Synthesis, antimicrobial activity, and determination of the lipophilicity of ((cyclohex-3-enylmethylene)hydrazinyl)thiazole derivatives. *Med. Chem. Res.* **2019**, *28(11)*, 2023-2036.
4. Wiegand I.; Hilpert K.; Hancock R.E.W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **2008**, *3(2)*, 163-175.
5. O'Donnell F.; Smyth T.J.; Ramachandran V.N.; Smyth W.F. A study of the antimicrobial activity of selected synthetic and naturally occurring quinolines. *Int. J. Antimicrob. Agents.* **2010**, *35*, 30-38.