Synthesis and Bioactivity Screening of Dihydroisoxazoles Derived from Eugenol

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Síntese e Avaliação da Bioatividade de Diidroisoxazóis Derivados do Eugenol

Resumo: Novos diidroisoxazóis (5a-d) derivado de eugenol foram sintetizados em bons rendimentos, e suas atividades antibacteriana, antifúngica e antioxidante foram avaliadas. Todos os diidroisoxazóis foram caracterizados usando análises elementar e espectral. Todos os compostos foram avaliados contra seis bactérias ATCC padrão: Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris and Proteus mirabilis. O composto mais ativo exibiu atividade antibacteriana contra E. coli (73 μg.mL⁻¹) e S. aureus (54 μg.mL⁻¹). Entretanto, nenhum dos compostos apresentou atividade contra as cepas de fungos (Candida albicans, Candida krusei, Candida parapsilosis and Aspergillus fumigatus) avaliadas. Todos os diidroisoxazóis sintetizados apresentaram melhores resultados como antioxidante comparado a referência (Trolox).

Palavras-chave: Diidroisoxazol; eugenol antibacteriano; antioxidante; antifúngico

Abstract

The four novel dihydroisoxazoles (5a-d) derived from eugenol were synthesized with good yields, and their antibacterial, antifungal and antioxidant activities were evaluated. All synthesized dihydroisoxazoles were characterized using spectral and elemental analyses. All compounds were tested against six standard ATCC bacteria: Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris and Proteus mirabilis. The most active compound exhibited antibacterial activity against E. coli (73 μg.mL⁻¹) and S. aureus (54 μg.mL⁻¹). However, none of the compounds showed activity against the fungus strains (Candida albicans, Candida krusei, Candida parapsilosis and Aspergillus fumigatus) assayed. All the synthesized dihydroisoxazoles presented better results as antioxidants than the reference (trolox).

Keywords: Dihydroisoxazole; eugenol; antibacterial; antioxidant; antifungal

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Synthesis and Bioactivity Screening of Dihydroisoxazoles Derived from Eugenol

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1. Introduction

Over the last decades, the synthesis and biological evaluation of heterocycles have played a pivotal role in modern drug design and medicinal chemistry research. The main interest in heterocyclic derivatives is associated with their diverse pharmacological activities. 1,2 Among the different azole derivatives the nucleus 4,5-dihydroisoxazole is an important class of five-membered heterocycles that possess...
a wide spectrum of biological properties, such as antibacterial, antifungal, analgesic, anti-inflammatory, antioxidant and antitumoral properties. This heterocycle is a significant pharmacophore that is present in the structure of a variety of pharmacologically active substances; for example, acivicin is an antineoplastic, antibiotic and anti-Leishmania drug that has been in commercial use for years (Figure 1).

Additionally, 4,5-dihydroisoxazole also exhibits important synthetic versatility to functionalize compounds, for example, β-hydroxy-ketones, α,β-unsaturated ketones and 1,3-amino-alcohol, which can be obtained from the reductive cleavage of the heterocyclic ring. These intermediates can be used for the synthesis of different organic compounds with biological activities. Although 4,5-dihydroisoxazole can be obtained by various different methods, the 1,3-dipolar cycloaddition reactions of nitrile oxides to alkenes is the most common.

The aryl-dihydroisoxazole moiety has attracted considerable interest for decades and represents an important scaffold in medical research field. This paper reports on the synthesis of novel dihydroisoxazoles using the cycloaddition reaction of substituted benzonitrile oxide to eugenol and the evaluation of the antibacterial, antioxidant and antifungal properties of these resultant dihydroisoxazoles.

2. Materials and Methods

The Fourier transform infrared (FTIR) spectra were recorded using a Hewlett-Packard FTIR spectrometer over a 4000-600 cm⁻¹ range. The FTIR samples were analyzed neat. ¹H and ¹³C NMR solution spectra were acquired on a Bruker Avance 500 spectrometer (500 MHz for ¹H and 125 MHz for ¹³C) with a 5 mm switchable probe at 22 ± 0.1 °C using a 12.5 μs and 7.0 μs pulses for ¹H and ¹³C, respectively. The spectra were acquired in chloroform-d (Cambridge Isotope Laboratories, Inc). The chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane, and the coupling constants (J) are expressed in Hertz (Hz). Multiplicities were presented as singlet (s), doublet (d), triplet (t), quadruplet (q) and multiplet (m). ¹H NMR spectra were acquired from 32 scans with a relaxation delay of 2.0 s, 16 K data points, and an 8,000 Hz spectral width using a digital resolution of 0.30 Hz. ¹³C NMR spectra were transformed after 38,644 scans with a 23,980.8 Hz spectral width using a digital resolution of 1 Hz and 32 K data points. Elemental analyses were performed in duplicate in a Perkin-Elmer 2400 with previously dried samples (45 °C, 24 h).

The mass spectra were recorded with a mass-selective Shimadzu GCMS-QP2010S detector.

![Figure 1. Examples of bioactive 4,5-dihydroisoxazole derivatives](image-url)
interacted with a capillary gas chromatograph. The analyses were performed using a DB-5ms capillary column (30 m X 0.25 mm) and film thicknesses of 0.5 μm. The column temperature was held at 40 °C for 4 minutes, increased to 280 °C at a rate of 10 °C.min⁻¹, and held at the latter temperature for 6 minutes. The injector and detector temperatures were 250 and 280 °C, respectively. The samples (1 μL) were injected in a split mode (ratio 1:20). Helium was used as the carrier gas at a flow rate of 1.0 mL.min⁻¹.

The progress of the reaction was monitored using thin layer chromatography (TLC) with silica gel-coated Merck 60 F254 plates, and the compounds were visualized with UV light (254 nm). Column chromatography was performed using Merck Kieselgel (0.040-0.063 mm) with ethyl acetate and hexane as the eluent. All reagents used for the different synthetic steps were purchased from Aldrich Chemical Co. and used as received. Antifungal assays used RPMI 1640 medium (Sigma), 3-(N-morpholino)-propane sulfonic acid (MOPS) buffer (Sigma), Sabouraud dextrose agar (Difco), Itraconazole (Sigma) and trichloroisocyanuric acid (TCIA) were added. The reaction medium remained in the ice bath for 15 minutes, after which it was maintained at room temperature for 1 h. Finally, the cyanuric acid formed was removed by filtration. This reaction was monitored by TLC using a mixture of hexane - ethyl acetate (5:1). This solution of imidoyl chloride was then used in the next step without any further treatment.

3.3. Cycloaddition of nitrile oxide to eugenol (5a-d)

Imidoyl chloride (30 mmol) from the previous reaction was slowly added to a solution of dichloromethane (20 mL) containing 30 mmol of eugenol and 30 mmol of Et₃N. The reaction was kept under constant stirring for 24 h at room temperature. After this period, the solvent was evaporated, and the product was purified by flash chromatography on silica gel (ethyl acetate:hexane 3:1). All dihydroisoxazoles were obtained as slightly yellow oils, with yields between 50 - 60 %.

4-[(3-phenyl)-4,5-dihydroisoxazol-5-ylmethyl]-2-methoxyphenol (5a)

Yield: 57 %; FTIR (cm⁻¹, neat – Figure S1): 3427 (ν C=O), 3063 (C-H), 2935-2842 (C sp3-H), 1600 (ν C=O), 1512 (C=C), 1265 (ν C-O), 1235 (ν C-CO), 1150 (ν C-O), 1032 (ν C-C) cm⁻¹; ¹³C NMR (500 MHz, CDCl₃, Figure S2) δ 156.7 (C10), 146.7 (C1), 144.6 (C2), 130.1 (C14), 129.9 (C11), 128.7 (C4), 128.6 (C12 and C16), 126.6 (C13 and C15), 122.1 (CS), 114.5 (C6), 112.1 (C3), 80.2 (C8), 56.0 (C17), 40.8 (C7), 39.4 (C9); GC-MS (Figure S4) - m/z: 283(M⁺); 138; 137; 118; 77; Elemental Anal. Calc. For C₁₇H₁₇NO₃C: 72.01; H, 6.41; N, 4.98. Found: C: 71.81; H: 6.03; N, 5.05.

4-[(3-bromophenyl)-4,5-dihydroisoxazol-5-ylmethyl]-2-methoxyphenol (5b)

Yield: 50 %; FTIR (cm⁻¹, neat – Figure S5): 3415 (ν C=O), 2931 (ν CH₃), 1555 (ν C=C), 1514 (C=C), 1271 (ν C-O), 1255 (ν C-CO), 1125 (ν C-O), 1034 (ν C-C) cm⁻¹; ¹H NMR (500 MHz, CDCl₃, Figure S6) δ 8.34 (s, 1H, H-12), 8.21 (dd, 1H, J 7.5 and 2.4 Hz, H-14), 8.01 (d,
1H, J 7.8 Hz, H-16), 7.59 (td, 1H, J 9.2 Hz and 3.5 Hz, H-15), 6.87 (d, 1H, J 8.6 Hz, H-6), 6.78 (s, 1H, H-3), 6.72 (d, 1H, J 8.6 Hz, H-5), 5.68 (s, 1H, OH), 5.10 – 5.03 (m, 1H, H-8), 3.87 (s, 3H, H-17), 3.36 (dd, 1H, J 16.5 and 10.1 Hz, H-9a), 7.15 (d, 1H, J 6.6 and 2.0 Hz, H-7b), 2.99 (dd, 1H, J 16.6 and 6.2 Hz, H-9b), 2.92 (dd, 1H, J 6.6 and 2.0 Hz, H-7b). 3H NMR (125 MHz, CDCl3 – Figure S7 and S8) δ 155.6 (C10), 146.7 (C1), 144.7 (C2), 132.0 (C14), 132.0 (C11), 130.9 (C12), 129.8 (C15), 128.7 (C4), 125.4 (C16), 122.9 (C5), 114.5 (C6), 112.0 (C3), 82.5 (C8), 55.7 (C17), 40.6 (C7), 38.8 (C9). GC-MS (Figure S9) m/z: 363(M+2), 361(Me+), 138, 137, 122; Elemental Anal. Calc. For C19H19NO5Br: C, 59.39; H, 4.79; N, 3.98. Found: C, 57.40; H, 4.82; N, 4.12.

4-[(3-nitrophenyl)-4,5-dihydroisoxazol-5-yl]methyl-2-methoxyphenol (5c)

Yield: 50%; FTIR (cm−1, neat – Figure S10): 3405(νC=O, C=N), 2935-2885 (Csp3-H), 1603 (νC=N), 1515 (C=C), 1270 (νC=N), 1237 (νC=O), 1182 (νC=O), 1032 (νC=O) cm−1; 1H NMR (500 MHz, CDCl3 – Figure S11) δ 8.37 (s, 1H, H-12), 8.24 (dd, 1H, J 8.4 and 1.4 Hz, H-14), 8.04 (d, 1H, J 8.4 Hz, H-16), 7.58 (t, 1H, J 8.0 Hz, H-15), 6.86 (d, 1H, J 8.4 Hz, H-6), 6.80 (d, 1H, J 2.0 Hz, H-3), 6.74 (dd, 1H, J 1.8 and 2.0 Hz, H-5), 5.50 (s, 1H, OH), 5.06 – 5.00 (m, 1H, H-8), 3.90 (s, 3H, H-17), 3.36 (dd, 1H, J 16.5 and 10.1 Hz, H-9a), 3.11-3.05 (m, 2H, H-9b), 2.90 (dd, 1H, J 13.8 and 6.6 Hz, H-7b). 13C NMR (125 MHz, CDCl3 – Figure S12 and S13) δ 155.3 (C10), 148.3 (C13), 146.6 (C11), 144.5 (C2), 132.5 (C16), 131.8 (C11), 130.3 (C15), 128.7 (C4), 124.5 (C14), 122.3 (C5), 121.3 (C12), 114.6 (C6), 112.1 (C3), 83.1 (C8), 56.2 (C17), 40.7 (C7), 39.3 (C9). GC-MS (Figure S16) m/z: 328 (M+), 138, 137, 107, 77; Elemental Anal. Calc. For C19H19NO5: C, 62.29; H, 4.94; N, 8.47. Found: C, 62.11; H, 4.94; N, 8.52.

4-[3-(3-methoxyphenyl)-4,5-dihydroisoxazol-5-y1)methyl]-2-methoxyphenol (5d)

Yield: 60%; FTIR (cm−1, neat – Figure S17): 3469 (νO-H), 2939-2845 (C=C-H), 1603 (νC=N), 1570 - 1515 (C=C), 1570 (νC=N), 1264 (νC=O), 1223 (νC=O), 1148 (νC=O), 1019 (νC=O) cm−1; 1H NMR (500 MHz, CDCl3 – Figure S18) δ 7.29 (t, 1H, J 7.9 Hz, H-15); 7.25 (s, 1H, H-12); 7.15 (d, 1H, J 7.9 Hz, H-16); 6.95 (d, 1H, J 7.2 Hz, H-14); 6.86 (d, 1H, J 7.9 Hz, H-6); 6.81 (s, 1H, H-3); 6.73 (d, 1H, J 7.9 Hz, H-5); 5.65 (s, 1H, OH); 5.00-4.96 (m, 1H, H-8); 3.88 (s, 3H, H-17); 3.83 (s, 3H, H-18); 3.30 (dd, 1H, J 16.4 and 10.3 Hz, H-9a); 3.10-3.02 (m, 2H, H-7a and H-9b); 2.84 (dd, 1H, J 14.0 and 6.6 Hz, H-7b). 13C NMR (125 MHz, CDCl3 – Figure S19) δ 159.6 (C10), 156.5 (C13), 146.7 (C1), 144.7 (C2), 131.0 (C11), 119.2 (C14), 128.9 (C4), 114.4 (C12), 129.9 (C15), 116.5 (C16), 121.9 (C5), 112.0 (C6), 111.3 (C3), 82.3(C8), 56.3 (C17), 55.3 (C18), 40.6 (C7), 39.3(C9). GC-MS (Figure S20) m/z: 313(M+), 176, 150, 138, 137. Elemental Anal. Calc. For C19H19NO5: C, 66.91; H, 6.14; N, 4.48. Found: C, 66.84; H, 6.14; N, 4.51.

3.4. Antimicrobial activity screening

Six standard strains of bacteria, namely, Staphylococcus aureus ATCC 25913, Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Proteus mirabilis ATCC 7002, and Proteus vulgaris ATCC 8427, which were preserved and activated at the Department of Microbiology of DCE/ENSP/FIOCRUZ, Rio de Janeiro, Brazil, were used in this study.

The dihydroisoxazoles (5a-d) were dissolved in tetrahydrofuran at concentration of 100 µg mL−1 to prepare extract stock solutions. The antimicrobial activity was determined using the agar diffusion assay as described by CLSI.18 The Mueller-Hinton agar (Merck) was used as the standard antimicrobial drug. Microorganisms were suspended in Brain Heart Infusion (BHI) (Difco, Detroit, MI, USA) broth and diluted to 106 colony forming units (CFU mL−1). They were inoculated onto the surface of BHI agar and Sabouraud Dextrose Agar (SDA, Difco) and then dried. The assays were performed in triplicate for each bacterium. Amoxicillin was used as the standard antimicrobial drug.

3.5. Antifungal activity screening

The Candida albicans (ATCC 10231), Candida krusei (ATCC 6258), Candida parapsilosis (ATCC 22019) and Aspergillus fumigatus (ATCC 16913) used in this study for antifungal screening were donated by INCQS/ Fiocruz (Instituto Nacional de Controle de Qualidade em Saúde), Rio de Janeiro, Brazil.

A microdilution broth method was used to determine the minimum inhibitory concentrations (MIC), according to the CLSI reference methods, documents M27-A3 and M38-A2, for yeasts and filamentous fungi, respectively.19,20

The assays were performed in 96-well culture microplates filled with RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (Sigma), 100 µL per well. Recent cultures,
in the log phase, of each strain were used to prepare the cell suspension adjusted to 1×10³ cells.mL⁻¹ for yeasts and 1×10⁴ cells.mL⁻¹ for filamentous fungi. The concentration of cells was confirmed by viable count on Sabouraud dextrose agar (Difco).

The samples were weighed, dissolved in 2 % DMSO and diluted in RPMI to 800 μg.mL⁻¹. All samples were tested at eight concentrations from 400 to 3.12 μg.mL⁻¹. The inoculum suspension (100 μL per well) was added to the microplates, which were incubated at 37 °C for 48 h. Itraconazole (Sigma) was used as the standard antifungal control (16 μg.mL⁻¹). Sterility and growth controls in the presence of organic solvents employed in sample preparation were also included. Resazurin (Sigma) solution (0.01 %) was added to each well, and the plates were incubated for 4 h at 37 °C. The MIC was defined as the lowest concentration of compounds that inhibited visible antifungal growth as indicated by the Resazurin colorimetric reagent. The tests were performed in triplicate.

3.6. Antioxidant activity screening

Antioxidant activity was measured by a spectrophotometric method using DPPH, as described in the literature. The dihydroisoxazoles (5a-d) were dissolved in DMSO, and on the day of the experiment, they were diluted from stock solutions to five different concentrations. An aliquot of each sample (10 μL) was added to 90 μL of ethanol and 50 μL of DPPH (0.3 mM). The mixture was placed in a dark room at 37 °C for 20 min, followed by measuring the absorbance using a plate reader at 517 nm. The standard reference was 6-hydroxy-2,5,7-tetramethylchroman-2-carboxylic acid (Trolox). The DPPH solution in the presence of DMSO was tested and used as a negative control.

4. Results and Discussion

4.1. Synthesis and characterization

The synthetic strategy in this work employed the pharmacophoric hybridization concept to design new dihydroisoxazoles derived from 4-allyl-2-methoxyphenol (eugenol) (Scheme 1). Eugenol was used due to its antimicrobial bioactivity described in the literature. The newly synthesized dihydroisoxazoles were characterized based on spectral data and screened for in vitro antimicrobial and antioxidant activities.

Based on the literature and our preliminary results, which revealed that meta-substituted aromatic aldehydes afforded bioactive dihydroisoxazoles, the main focus was to synthesize novel 3,5-disubstituted dihydroisoxazoles derived from eugenol. These hybrid compounds (5a-d) were synthesized from different aromatic aldehydes (1a-d, which were treated with hydroxylamine hydrochloride in ethanol to produce the respective aldoximes. Halogenated derivatives (3a-d) were obtained from the reaction of aldoximes with trichloroisocyanuric acid (TCIA) in Et₃N/CH₂Cl₂.

![Scheme 1. Synthesis of dihydroisoxazole derivatives (5a-d)](image-url)
The dihydroisoxazoles (5a-d) were obtained in a single step via a 1,3-dipolar cycloaddition reaction using imidoyl chlorides 3a-d as the starting materials, which were subsequently added to a mixture of eugenol (4) and Et$_3$N in dichloromethane (Scheme 1).

The reactions were monitored by TLC on silica gel plates, and the products were purified by flash chromatography. Structures 5a-d were confirmed by FTIR, GC-MS, NMR techniques and elemental analysis.

The FTIR spectra of dihydroisoxazoles (5a-d) showed absorptions at approximately 1035-1019 cm$^{-1}$ and 1600-1560 cm$^{-1}$, which were attributed to $\nu_{C-O}$ and $\nu_{C=N}$ stretches. The mass spectra of dihydroisoxazoles (5a-d) presented values of the molecular ion peak compatible with the expected structure. Additionally, a fragment at m/z 137 (100 %) was observed, which was related to fragmentation at the benzylic position of the eugenol moiety (Figure 2).

Different NMR techniques ($^1$H, $^{13}$C and 2D NMR) were used to confirm the structures obtained (Figure S1-S20 – supplemental material). The assignment of the regiochemistry in 4,5-dihydroisoxazole (5a-d) was unequivocally made on the basis of the $^1$H and $^{13}$C NMR spectra of the ring moiety. The $^1$H NMR spectra showed the 4,5-dihydroisoxazole ring protons, H-9 and H-8, in the 3.00 - 5.10 ppm region. The analysis showed that the methylene H-9a and H-7b signals were two different doublet of doublets around $\delta$ 3.30 ppm ($J = 16.5$ and 10.1 Hz) and $\delta$ 2.90 ppm ($J = 13.5$ and 6.6 Hz), respectively, while the H-9b and H-7a hydrogens were overlapped in a multiplet around $\delta$ 3.13 – 3.01 ppm. The methine hydrogen H-8 signal appeared as a multiplet around $\delta$ 5.10 – 4.92 ppm.

The $^{13}$C NMR spectra for these four novel products showed signals around 156, 82 and 39 ppm attributed to C-10, C-8 and C-9, respectively. Additionally, the C-9 and C-8 signals for the 5c APT spectrum (supplemental material) had positive and negative amplitudes, respectively. These results confirmed that 4,5-dihydroisoxazole was 3,5-disubstituted.

The regiosselectivity observed is probably due a preferential spatial arrangement of the reactive intermediates in the transition state. This result can be predicted by the frontier molecular orbital theory which demonstrated that, in some cases, the most substituted carbon will bind to the oxygen of the nitrile oxide as a consequence of the HOMO-LUMO interaction of the reagents.

Analysis of $^1$H, $^{13}$C NMR and the two dimensional spectra (HSQC and HMBC - supplemental material) provided the identification of aromatic rings. In the HSQC spectrum, the aromatic hydrogens of the eugenol moiety, H-3 ($\delta$ 6.80 ppm, $d$, $J = 2.0$ Hz), H-5 ($\delta$ 6.74 ppm, $dd$, $J = 8.4$ and 2.0 Hz) and H-6 ($\delta$ 6.86 ppm, $d$, $J = 8.4$ Hz) showed cross signals with carbons C-3 ($\delta$ 112.1 ppm), C-5 ($\delta$ 122.3 ppm) and C-6 ($\delta$ 114.6 ppm). The quaternary carbons C-1 ($\delta$ 146.6 ppm), C-2 ($\delta$ 144.5 ppm) and C-4 ($\delta$ 128.7 ppm) were determined from the APT and HMBC spectra. The hydroxyl and methyl (H-17) groups showed signals, in $^1$H NMR spectrum, as singlet around $\delta$ 5.50 and $\delta$ 3.90 ppm for all 4,5-dihydroisoxazoles, respectively.

The $^1$H NMR data of 5a-d, also, showed signals compatible with meta-substituted homoaromatic ring, which were influenced by the electronic characteristics of the substituents. For example, the homoaromatic moiety of 5c that contained the nitro group showed the most unshielded signals. The singlet at $\delta$ 8.37 ppm and doublet of doublets at $\delta$ 8.24 ppm ($J = 7.8$ Hz) represent the hydrogens H-12 and H-14, respectively. The triplet at $\delta$ 7.58 ppm

Figure 2. Fragmentation route to the ion m/z 137 for 5a
(J = 8.0 Hz) and duplet at δH 8.04 ppm (J = 8.4 Hz) represent the hydrogens H-15 and H-16. The proposed assignments were confirmed by APT and HSQC spectra (supplemental material). In the HSQC spectrum the hydrogens H-12, H-14, H-15 and H-16 showed correlations to δc 121.3, 124.5, 130.3 and 132.5 ppm, respectively. Finally, in the HMBC spectrum correlations observed from δH 8.37 (H-12) to δc 131.8 ppm suggested that was C-11. Additionally, correlations from H-12 and H-14 to δc 148.3 ppm indicated that was C-13.

4.2. Antibacterial activity

The potential bioactivity was assessed for all products. First, the antimicrobial activities of 5a-d were evaluated against two Gram-positive (Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212) and four Gram-negative (Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Proteus mirabilis ATCC 7002 and Proteus vulgaris ATCC 8427) microorganisms using an agar dilution procedure. Compounds 5b, 5c and 5d did not show any microbial inhibition activity on the strains tested (Table 1). The results observed for eugenol were consistent with those in the literature, which reports that eugenol presents some antimicrobial activity at high concentrations (≥ 500.00 μg.mL⁻¹). 5c showed activity against S. aureus, with MIC values of 80 and 54 μg.mL⁻¹, respectively (Table 1). Compound 5c (MIC - 73 μg.mL⁻¹) exhibited moderate bioactivity compared to the reference (amoxicillin – 16 μg.mL⁻¹) against P. aeruginosa.

4.3. Antifungal activity

All dihydroisoxazoles produced in this work were evaluated against four strains of fungi: Aspergillus fumigatus, Candida albicans, Candida krusei, and Candida parapsilosis. Compounds 5b, 5c and 5d exhibited some antifungal activity against Candida albicans only at high concentrations (≥ 200 μg.mL⁻¹), although 5b and 5c were active against Aspergillus Fumigatus (MIC – 100 μg.mL⁻¹) (Table 2).

4.4. Antioxidant activity

The antioxidant capacities of the dihydroisoxazoles synthesized here were determined using the DPPH scavenging method. Eugenol (4) exhibited discrete antioxidant activity, whereas the other products exhibited comparable or slightly less radical-scavenging ability than the standard (Trolox) at concentrations of 300 μM and 150 μM (Figure 3). At lower concentrations, 5a (R = H), 5b (R = Br) and 5d (R = OCH₃) exhibited higher activities than the standard (Figure 3). The 5c compound, which contains an electron-withdrawing substituent (nitro group), exhibited a lower scavenging activity. The antioxidant activity of these organic molecules is influenced by the nature of the substituent groups. In this case, compounds 5a, 5b and 5d showed the highest scavenging activity (DPPH method) with IC₅₀ at concentrations of 64, 41 and 63 μM, respectively (Figure 3). Overall, the newly synthesized compounds exhibited better scavenging activity than eugenol, (4) (IC₅₀ 83 μM).

Table 1. In vitro antibacterial activity (MIC) of the synthetized compoundsa.

| Bacteria        | 4,5-Dihydroisoxazoles | Amoxicillinb |
|-----------------|-----------------------|--------------|
|                 | 5a                    | 5b | 5c | 5d | 4 |
| S. aureus       | 80                    | +  | 54 | +  | 4 |
| E. faecalis     | +                     | +  | +  | +  | - |
| P. aeruginosa   | +                     | +  | 73 | +  | 16|
| E. coli         | +                     | +  | +  | +  | - |
| P. mirabilis    | +                     | +  | +  | +  | - |
| P. vulgaris     | 80                    | +  | 54 | +  | 4 |

a Minimum inhibitory concentration in μg.mL⁻¹; b Amoxicillin - used as a standard reference only when the compounds exhibited some activity; (+) denotes bacterial growth and (-) denotes not evaluated.
Table 2. Antifungal activities of 4,5-dihydroisoxazoles.

| Compound | Candida Albicans | Candida Krusei | Candida parapsilosis | Aspergillus Fumigatus |
|----------|------------------|---------------|----------------------|-----------------------|
| 4        | +                | +             | +                    | +                     |
| 5a       | +                | +             | +                    | +                     |
| 5b       | 200              | +             | +                    | 100                   |
| 5c       | 400              | +             | 400                  | 100                   |
| 5d       | 200              | +             | +                    | +                     |

* Minimum inhibitory concentration in μg.mL⁻¹; (+) denotes growth of fungi. ²Itraconazole was used as positive control (16 μg.mL⁻¹)

5. Conclusion

These four novel 4,5-dihydroisoxazoles, which were synthesized regiospecifically with moderate yields (50 – 60 %), were produced using a simple methodology and with inexpensive reagents. The 5c dihydroisoxazole was the most efficient against *Staphylococcus aureus* and *Proteus vulgaris* (MIC 54 μg.mL⁻¹, for both), although less efficient than amoxicillin (MIC = 4 μg.mL⁻¹). All dihydroisoxazoles showed some antifungal activity but only at high concentrations (100 – 400 μg.mL⁻¹). However, these novel compounds proved to be interesting antioxidant agents.

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