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Article

Synthesis, Antioxidant Activity, Acetylcholinesterase Inhibition and Quantum Studies of Thiosemicarbazones

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Thiosemicarbazones are a class of compounds of interest for Medicinal Chemistry, as they are structurally diverse and have numerous biological activities reported in the literature. This study describes the synthesis of seventeen thiosemicarbazones, which were investigated as potential therapeutic agents for the treatment of Alzheimer's disease through antioxidant tests and an inhibitory assay of the acetylcholinesterase enzyme. All compounds showed excellent inhibition of acetylcholinesterase and exhibited excellent antioxidant action when compared to the standards. In addition, a quantum study was carried out, in which the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) energy values of each compound were obtained. From these theoretical data, chemical properties were calculated and correlated with the experimental data.

Keywords: antioxidants, acetylcholinesterase, Alzheimer's disease, thiosemicarbazone

Introduction

Free radicals are reactive molecules containing an unpaired electron or an odd number of electrons.[1] They can be formed by redox reactions, the cleavage of a radical to form another or the breaking of a chemical bond, each of these conditions being able to generate a fragment that maintains an unpaired electron. Examples of free radicals are hydroxyl ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$).[2] In addition, there are other reactive species that can oxidize compounds, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and species that can easily form radicals, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$).[3]

Free radicals and ROS are present in biological systems and can be generated from a variety of sources. These reactive species are able to oxidize proteins, lipids or nucleic acids, and, thereby, have the potential to initiate oxidative stress and cause damage to cell structures, being, in turn, linked to chronic and degenerative diseases,[4] such as cancer,[5] cardiovascular diseases,[6] alcohol-induced liver disease,[7] atherosclerosis,[8] neural disorders,[9] and Alzheimer's disease,[10,11] and also aging processes.[12]

Oxidative stress appears as a risk factor for age-associated cognitive decline and is considered to be a critical aspect in the pathogenesis of Alzheimer's disease (AD).[13] AD is a progressive neurodegenerative disorder of the central nervous system characterized by mental deterioration.[14] The disease is not completely understood, different hypotheses try to explain its causes and form the basis of current therapies.[15] Treatment strategies employ the use of acetylcholinesterase inhibitors, glutamate receptor modulators, and antioxidants and anti-inflammatory agents.[16,17] Nowadays, the cholinergic hypothesis is the most accepted explanation of the cause of AD[13] since the disease is associated with a reduction in the levels of the acetylcholine (ACh) neurotransmitter in areas of the brain that deal with behavior, learning, memory and emotional responses.[18] Acetylcholinesterase (AChE) is one of the enzymes responsible for ACh hydrolysis, thus controlling ACh concentration in the organism.[15] For this reason, current drugs for AD aim to inhibit AChE, such as galantamine, rivastigmine, tacrine, ensaculin, and donepezil.[18]

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Another interesting hypothesis proposes that AD is related to oxidative stress, with several studies showing that oxidative damage is linked to AD, and more recent studies suggesting that it is associated with the earlier stages of the disease.[19,20] The brain is especially susceptible to oxidative damage because it has limited antioxidant capacity, high lipid content and high oxygen consumption rate when compared to other organs.[11,20] Thereby, the use of antioxidant compounds have produced promising results in relation to AD.[21] The main characteristic of an antioxidant is its ability to trap free radicals; antioxidant compounds scavenge free radicals and thus inhibit the oxidative mechanisms that lead to degenerative diseases.[4]

The existence of different possible hypotheses to explain the onset and progression of AD directs the development of new drugs toward molecules with multiple actions that are able to act in a complementary manner and could bring satisfactory results for the treatment of the disease.[11,22]

There is considerable interest in thiosemicarbazones because they constitute an important class of compounds whose properties have been extensively studied in Medicinal Chemistry,[23] displaying a wide range of biological activities, such as antiviral,[24] antitumor,[25] antimicrobial,[26] antibacterial and antifungal[27] properties.

Moreover, studies show that these compounds have excellent antioxidant activities[27,28] and other authors have reported interesting results of thiosemicarbazones in combating AD.[29-31] Thiosemicarbazones are reported to act as enzyme inhibitors by complexing endogenous metals or by redox reactions and as inhibitors of DNA synthesis through DNA interactions.[23] Furthermore, thiosemicarbazones have been proposed to inhibit cysteine proteases, which are essential to several functions of the malaria parasite.[32]

Based on these observations, the aim of this study was to synthesize a series of thiosemicarbazones, four of which had not been reported in the literature before, as potential therapeutic agents for the treatment of AD. The compounds were assayed to investigate their ability to inhibit acetylcholinesterase (AChE), and two *in vitro* antioxidants tests were performed, evaluating the thiosemicarbazones' nitric oxide (NO) scavenging activity and hydrogen peroxide (H_2O_2) scavenging activity. In addition, quantum studies were performed to theoretically analyze the compounds' antioxidant activity.

Experimental

General procedure

All reagents were obtained commercially (Sigma-Aldrich), and all solvents used were of analytical grade, without additional purification. The obtained compounds were analyzed by thin layer chromatography (TLC) using aluminum plates coated with silica gel on TLC Al foils (Sigma-Aldrich). All synthesized compounds were characterized by melting points (mp), infrared (IR) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). The structures of all compounds were confirmed by mass spectrometry. Melting points were determined with a Microquimica MGAPF-301 apparatus. Infrared spectra were obtained on FTIR spectrophotometer with a Fourier transform and accessory attenuated total reflection (FTIR-ATR) PerkinElmer Spectrophotometer Spectrum 100 with a crystal of ZnSe (45°) and a TGS (triglycine sulfate) detector, the samples were analyzed directly on the crystal by the attenuated total reflection, averaging 18 scans in the range 4000-400 cm^{-1} and a resolution of 4 cm^{-1}. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Ac-200F (operating at 200 MHz for ^{1}H and 50 MHz for ^{13}C) or a Bruker Avance Drx 400 spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C), with tetramethylsilane as internal standard.

High-resolution mass spectra (HRMS) were recorded on a micrOTOF-QII (Bruker Daltonics) mass spectrometer, equipped with an automatic syringe pump (KD Scientific) for sample injection (constant flow of 3 μL min^{-1}), by positive mode of electron spray ionization (ESI-MS) technique (4.5 kV and 180 °C) using acetonitrile as solvent. The instrument was calibrated in the range *m/z* 50-3000 using an internal calibration standard (low concentration tuning mix solution), supplied by Agilent Technologies. Data was processed via Bruker Data Analysis software (version 4.0). When the calculated and experimental masses were compared, the error was as expected (< 2 ppm). Spectrophotometric measurements were performed on a DU640 spectrophotometer from Beckman Instruments, Inc. (Fullerton, CA).

Procedure for the synthesis of *N*-(*p*-tolyl)hydrazinecarbothioamide (**1**)

Compound **1** was synthesized by reacting 1-isothiocyanato-4-methylbenzene (10 mmol) and hydrazine hydrate (50%, 20 mmol) in 20 mL of isopropanol under magnetic stirring for 3 h at room temperature. The precipitate formed was separated by vacuum filtration and washed with isopropanol to obtain the pure product.[33]

N-(*p*-Tolyl)hydrazinecarbothioamide (**1**)

Yield 52%; white solid; mp 137.7-138.3 °C; IR (ATR) ν / cm^{-1} 3294 (NH), 3242 and 3190 (NH_2), 1200 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.39 (d, 2H, *J* 7.8 Hz), 7.17

(d, 2H, *J* 8.3 Hz), 3.99 (s, 2H, NH_2), 2.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.0, 135.9, 135.1, 129.4, 124.6, 21.0; HRMS (ESI-TOF) *m/z*: 182.0745 $[M + H]^+$, calculated for $C_8H_{11}N_3S$, 182.0746.

General procedure for the synthesis of thiosemicarbazones (**2a-q**)

Thiosemicarbazones **2a-q** were prepared by the stirring and refluxing of a solution containing: *N*-(*p*-tolyl) hydrazinecarbothioamide (1.19 mmol), appropriately substituted benzaldehyde (1.25 mmol), 11 mL of ethanol and 22 mL of water with a catalytic amount of acetic acid. After one hour, the mixture was cooled down to ambient temperature, and the precipitate formed was collected with vacuum filtration and washed with water.[26]

Data referencing the characterization of compounds **2a-2q** can be analyzed subsequently.

(*E*)-2-Benzylidene-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2a**)

Yield 81%; white solid; mp 171.0-172.0 °C; IR (ATR) ν / cm^{-1} 3300 and 3148 (NH), 1589 (C=N), 1201 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 10.15 (s, 1H, NH), 9.12 (s, 1H, NH), 7.95 (s, 1H, -NC*H*), 7.68 (m, 2H), 7.50 (d, 2H, *J* 8.2 Hz), 7.42 (m, 3H), 7.22 (d, 2H, *J* 8.2 Hz), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 142.9, 136.2, 135.2, 133.1, 130.6, 129.4, 128.8, 127.4, 124.8, 21.0; HRMS (ESI-TOF) *m/z*: 270.1063 $[M + H]^+$, calculated for $C_{15}H_{15}N_3S$, 270.1059.

(*E*)-2-(4-Methylbenzylidene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2b**)

Yield 80%; white solid; mp 195.8-196.5 °C; IR (ATR) ν / cm^{-1} 3302 and 3127 (NH), 1602 (C=N), 1190 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 9.73 (s, 1H, N*H*), 9.11 (s, 1H, N*H*), 7.87 (s, 1H, -NC*H*), 7.57 (d, 2H, *J* 8.1 Hz), 7.50 (d, 2H, *J* 8.1 Hz), 7.22 (d, 4H, *J* 8.0 Hz), 2.39 (s, 3H), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.0, 143.2, 141.1, 136.2, 135.2, 130.4, 129.6, 129.4, 127.4, 124.9, 21.5, 21.0; HRMS (ESI-TOF) *m/z*: 284.1213 $[M + H]^+$, calculated for $C_{16}H_{17}N_3S$, 284.1216.

(*E*)-2-(4-Nitrobenzylidene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2c**)

Yield 97%; yellow solid; mp 207.5-209.5 °C; IR (ATR) ν / cm^{-1} 3306 and 3127 (NH), 1592 (C=N), 1177 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 9.99 (s, 1H, N*H*), 9.08 (s, 1H, N*H*), 8.29 (d, 2H, *J* 8.8 Hz), 7.96 (s, 1H, -NC*H*), 7.85 (d, 2H, *J* 8.8 Hz), 7.50 (d, 2H, *J* 8.3 Hz), 7.22 (d, 2H), 2.39 (s, 3H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 177.0, 148.1, 141.0, 140.4, 136.8, 135.2, 129.0, 128.9, 126.5, 124.2, 21.0; HRMS (ESI-TOF) *m/z*: 315.0913 $[M + H]^+$, calculated for $C_{15}H_{14}N_4O_2S$, 315.0910.

(*E*)-2-(4-Chlorobenzylidene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2d**)

Yield 93%; white solid; mp 172.5-174.4 °C; IR (ATR) ν / cm^{-1} 3328 and 3306 (NH), 1593 (C=N), 1192 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 9.99 (s, 1H, N*H*), 9.07 (s, 1H, N*H*), 7.88 (s, 1H, -NC*H*), 7.62 (d, 2H, *J* 8.6 Hz), 7.49 (d, 2H, *J* 8.3 Hz), 7.39 (d, 2H, *J* 8.6 Hz), 7.22 (d, 2H, *J* 8.2 Hz), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 141.4, 136.6, 136.4, 135.1, 131.6, 129.4, 129.2, 128.5, 124.9, 21.0; HRMS (ESI-TOF) *m/z*: 304.0672 $[M + H]^+$, calculated for $C_{15}H_{14}ClN_3S$, 304.0670.

(*E*)-2-(4-Methoxybenzylidene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2e**)

Yield 82%; white solid; mp 183.2-185.0 °C; IR (ATR) ν / cm^{-1} 3317 and 3142 (NH), 1607 (C=N), 1194 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 9.98 (s, 1H, N*H*), 9.10 (s, 1H, N*H*), 7.88 (s, 1H, -NC*H*), 7.63 (d, 2H, *J* 8.7 Hz), 7.51 (d, 2H, *J* 8.2 Hz), 7.22 (d, 2H, *J* 8.1 Hz), 6.94 (d, 2H, *J* 8.7 Hz), 3.86 (s, 3H, *p*-OCH_3), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.8, 161.7, 142.9, 136.1, 135.3, 129.4, 129.1, 125.8, 124.9, 114.3, 55.4, 21.0; HRMS (ESI-TOF) *m/z*: 300.11653 $[M + H]^+$, calculated for $C_{16}H_{17}N_3OS$, 300.11651.

(*E*)-2-(Benzo[*d*][1,3]dioxol-5-ylmethylene)-*N*-(*p*-tolyl) hydrazinecarbothioamide (**2f**)

Yield 60%; white solid; mp 183.0-184.8 °C; IR (ATR) ν / cm^{-1} 3305 and 3178 (NH), 1591 (C=N), 1184 (C=S); ^1H NMR (200 MHz, DMSO-d_6) δ 11.66 (s, 1H, N*H*), 10.02 (s, 1H, N*H*), 8.05 (s, 1H, -NC*H*), 7.83 (s, 1H), 7.39 (d, 2H, *J* 8.2 Hz), 7.18-7.12 (m, 3H), 6.94 (d, 1H, *J* 7.5 Hz), 6.08 (s, 2H), 2.31 (s, 3H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 176.3, 149.5, 148.5, 143.0, 137.0, 134.9, 129.1, 128.9, 126.4, 124.7, 108.6, 106.1, 101.9, 21.0; HRMS (ESI-TOF) *m/z*: 314.0956 $[M + H]^+$, calculated for $C_{16}H_{15}N_3O_2S$, 314.0958.

(*E*)-2-([1,1'-Biphenyl]-4-ylmethylene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2g**)

Yield 82%; white solid; mp 165.9-167.8 °C; IR (ATR) ν / cm^{-1} 3303 and 3146 (NH), 1591 (C=N), 1187 (C=S); ^1H NMR (200 MHz, $CDCl_3$) δ 10.77 (s, 1H, N*H*), 9.15 (s, 1H, N*H*), 8.03 (s, 1H, -NCH), 7.72 (d, 2H, *J* 8.4 Hz), 7.63-7.58 (m, 4H), 7.49 (m, 4H, *J* 8.4 and 8.4 Hz), 7.41-7.36 (m, 1H), 7.21 (d, 2H, *J* 8.0 Hz), 2.36 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.9, 143.3, 142.9, 140.1, 136.2, 135.2, 132.1, 129.4, 128.9, 127.9, 127.5, 127.0, 125.0, 21.1;

HRMS (ESI-TOF) *m/z*: 346.1369 $[M + H]^+$, calculated for $C_{21}H_{19}N_3S$, 346.1372.

(*E*)-2-(4-Hydroxy-3-methoxybenzylidene)-*N*-(*p*-tolyl) hydrazinecarbothioamide (**2h**)

Yield 83%; white solid; mp 157.5-159.0 °C; IR (ATR) ν / cm^{-1} 3515 (OH), 3394 and 3250 (NH), 1602 (C=N), 1192 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.37 (s, 1H, N*H*), 9.05 (s, 1H, N*H*), 7.89 (s, 1H, -NC*H*), 7.47 (d, 2H, *J* 8.4 Hz), 7.24-7.14 (m, 4H), 6.93 (d, 1H, *J* 8.0 Hz), 5.93 (s, 1H), 3.94 (s, 3H, *m*-OCH_3), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.8, 148.3, 146.9, 143.6, 136.3, 135.3, 129.4, 125.6, 125.2, 122.7, 114.7, 108.5, 56.1, 21.0; HRMS (ESI-TOF) *m/z*: 316.1113 $[M + H]^+$, calculated for $C_{16}H_{17}N_3O_2S$, 316.1114.

(*E*)-*N*-(*p*-Tolyl)-2-(3,4,5-trimethoxybenzylidene)hydrazine-carbothioamide (**2i**)

Yield 73%; white solid; mp 167.8-169.9 °C; IR (ATR) ν / cm^{-1} 3298 and 3140 (NH), 1577 (C=N), 1193 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.36 (s, 1H, N*H*), 9.04 (s, 1H, N*H*), 7.88 (s, 1H, -NC*H*), 7.48 (d, 2H, *J* 8.2 Hz), 7.23 (d, 2H, *J* 8.4 Hz), 6.88 (s, 2H), 3.91 (s, 6H, *m*-OCH_3), 3.89 (s, 3H, *p*-OCH_3), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 153.6, 143.1, 140.6, 136.5, 135.2, 129.5, 128.4, 125.3, 104.7, 61.0, 56.3, 21.0; HRMS (ESI-TOF) *m/z*: 360.1373 $[M + H]^+$, calculated for $C_{18}H_{21}N_3O_3S$, 360.1376.

(*E*)-2-(Naphthalen-1-ylmethylene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2j**)

Yield 83%; light yellow solid; mp 173.0-175.0 °C; IR (ATR) ν / cm^{-1} 3305 and 3206 (NH), 1593 (C=N), 1189 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.28 (s, 1H, N*H*), 9.18 (s, 1H, N*H*), 8.70 (s, 1H, -NC*H*), 8.41 (dd, 1H, *J* 9.0 and 2.5 Hz), 7.97-7.89 (m, 3H), 7.60-7.48 (m, 5H), 7.21 (d, 2H, *J* 8.2 Hz), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 141.9, 136.2, 135.5, 133.8, 131.2, 130.9, 129.4, 129.0, 128.8, 127.4, 126.9, 126.3, 125.3, 124.7, 123.2, 21.0; HRMS (ESI-TOF) *m/z*: 320.12157 $[M + H]^+$, calculated for $C_{19}H_{17}N_3S$, 320.12159.

(*E*)-2-(Naphthalen-2-ylmethylene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2k**)

Yield 73%; white solid; mp 199.3-200.3 °C; IR (ATR) ν / cm^{-1} 3311 and 3140 (NH), 1593 (C=N), 1196 (C=S); ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.25 (s, 1H, N*H*), 9.18 (s, 1H, N*H*), 8.10 (s, 1H, -NC*H*), 7.96-7.82 (m, 5H), 7.55-7.50 (m, 4H), 7.23 (d, 2H, *J* 8.6 Hz), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.0, 143.2, 136.3, 135.2, 134.4, 133.1, 130.7, 129.8, 129.4, 128.7, 128.4, 127.9, 127.4, 126.8, 125.0, 122.4, 21.1; HRMS (ESI-TOF) *m/z*: 320.1214 $[M + H]^+$, calculated for $C_{19}H_{17}N_3S$, 320.1216.

(*E*)-2-(3,4-Dichlorobenzylidene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2l**)

Yield 76%; white solid; mp 206.4-208.0 °C; IR (ATR) ν / cm^{-1} 3345 and 3127 (NH), 1590 (C=N), 1270 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.54 (s, 1H, N*H*), 9.04 (s, 1H, N*H*), 7.89 (s, 1H, -NC*H*), 7.77 (s, 1H), 7.50-7.45 (m, 4H), 7.23 (d, 2H, *J* 8.2 Hz), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.2, 140.2, 136.6, 134.9, 134.6, 133.4, 133.2, 130.9, 129.5, 128.6, 126.5, 125.1, 21.1; HRMS (ESI-TOF) *m/z*: 338.02801 $[M + H]^+$, calculated for $C_{15}H_{13}Cl_2N_3S$, 338.02800.

(*E*)-2-(2,5-Dimethoxybenzylidene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2m**)

Yield 87%; white solid; mp 179.0-181.2 °C; IR (ATR) ν / cm^{-1} 3294 and 3143 (NH), 1594 (C=N), 1217 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.20 (s, 1H, N*H*), 9.08 (s, 1H, N*H*), 8.22 (s, 1H, -NC*H*), 7.51 (d, 2H, *J* 8.4 Hz), 7.38 (d, 1H, *J* 2.9 Hz), 7.20 (d, 2H, *J* 8.2 Hz), 6.97 (dd, 1H, *J* 9.0 and 3.0 Hz), 6.87 (d, 1H, *J* 9.1 Hz), 3.84 (s, 3H, *o*-OCH_3), 3.82 (s, 3H, *m*-OCH_3), 2.36 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 153.7, 153.1, 138.8, 136.0, 135.3, 129.3, 124.7, 122.1, 117.7, 112.6, 110.6, 56.2, 55.9, 21.0; HRMS (ESI-TOF) *m/z*: 330.1274 $[M + H]^+$, calculated for $C_{17}H_{19}N_3O_2S$, 330.1271.

(*E*)-2-(3,4-Dimethoxybenzylidene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2n**)

Yield 83%; white solid; mp 182.5-184.5 °C; IR (ATR) ν / cm^{-1} 3329 and 3138 (NH), 1601 (C=N), 1135 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.93 (s, 1H, N*H*), 9.04 (s, 1H, N*H*), 7.86 (s, 1H, -NC*H*), 7.49 (d, 2H, *J* 8.2 Hz), 7.25-7.16 (m, 4H), 6.89 (d, 1H, *J* 8.2 Hz), 3.94 and 3.93 (s, 6H, *m*-OCH_3 and *p*-OCH_3), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.9, 151.5, 149.4, 143.3, 136.3, 135.3, 129.4, 126.0, 125.1, 122.4, 111.0, 109.0, 56.0, 21.0; HRMS (ESI-TOF) *m/z*: 330.1270 $[M + H]^+$, calculated for $C_{17}H_{19}N_3O_2S$, 330.1271.

(*E*)-2-(2-Nitrobenzylidene)-*N*-(*p*-tolyl)hydrazinecarbo-thioamide (**2o**)

Yield 90%; yellow solid; mp 194.3-195.7 °C; IR (ATR) ν / cm^{-1} 3281 and 3125 (NH), 1535 (C=N), 1188 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.72 (s, 1H, N*H*), 9.06 (s, 1H, N*H*), 8.42 (s, 1H, -NC*H*), 8.09-8.00 (m, 2H), 7.74-7.50 (m, 4H), 7.21 (d, 2H, *J* 8.4 Hz), 2.37 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.4, 148.2, 137.6, 136.3, 135.0, 133.3, 130.6, 129.4, 128.7, 128.1, 125.0, 124.4, 21.0;

HRMS (ESI-TOF) *m/z*: 315.0907 $[M + H]^+$, calculated for $C_{15}H_{14}N_4O_2S$, 315.0910.

(*E*)-2-((5-(2-Chloro-5-(trifluoromethyl)phenyl)furan-2-yl) methylene)-*N*-(*p*-tolyl)hydrazinecarbothioamide (**2p**)

Yield 92%; light yellow solid; mp 207.5-209.5 °C; IR (ATR) ν / cm^{-1} 3344 and 3140 (NH), 1621 (C=N), 1266 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.96 (s, 1H, N*H*), 9.19 (s, 1H, N*H*), 8.20 (s, 1H, -NC*H*), 7.83 (s, 1H), 7.62-7.46 (m, 4H), 7.33 (d, 1H, H3', *J* 3.7 Hz), 7.23 (d, 2H, *J* 8.5 Hz), 6.92 (d, 1H, H2', *J* 3.7 Hz), 2.38 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.1, 150.7, 149.5, 136.8, 134.9, 133.6, 132.6, 132.1, 129.9, 129.3, 129.0, 128.6, 128.0, 126.7, 126.0, 125.4, 125.0, 121.3, 115.2, 114.9, 21.0; HRMS (ESI-TOF) *m/z*: 438.0650 $[M + H]^+$, calculated for $C_{20}H_{15}ClF_3N_3OS$, 438.0649.

(*E*)-2-(4-(Dimethylamino)benzylidene)-*N*-(*p*-tolyl)hydrazine-carbothioamide (**2q**)

Yield 89%; yellow solid; mp 195.2-196.4 °C; IR (ATR) ν / cm^{-1} 3290 and 3140 (NH), 1588 (C=N), 1265 (C=S); ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.45 (s, 1H, N*H*), 9.09 (s, 1H, N*H*), 7.77 (s, 1H, -NC*H*), 7.57-7.50 (m, 4H), 7.20 (d, 2H, *J* 8.3 Hz), 6.69 (d, 2H, *J* 8.9 Hz), 3.03 (s, 6H), 2.36 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.4, 152.0, 143.8, 135.8, 135.6, 129.3, 129.0, 124.7, 120.6, 111.8, 40.1, 21.0; HRMS (ESI-TOF) *m/z*: 313.1479 $[M + H]^+$, calculated for $C_{17}H_{20}N_4S$, 313.1481.

Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was measured by the methodology described by Chandrashekaraiah *et al.*[34] In this reaction, nitric oxide radicals ($NO^•$) are generated from sodium nitroprusside and interact with oxygen to produce nitrite ions that can be estimated using a Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). To measure the nitric oxide scavenging activity, 1 mL of sodium nitroprusside (10 mM) and 1.5 mL of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (25, 50, 75 and 100 mg mL^{-1}) of the test compounds and incubated for 150 min at 25 °C, and then 1 mL of the reaction mixture was treated with 1 mL of Griess reagent. The absorbance (A) was measured at 546 nm and nitric oxide scavenging activity was calculated using equation 1.[34] The results of antioxidant assays were expressed as EC_{50}.

$$\% \text{ of scavenging} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance without the sample; A_{sample} is the absorbance of the sample.

Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging ability of the test compounds was determined spectrophotometrically, according to the method described by Ruch *et al.*[35] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). 25, 50, 75 and 100 mg mL^{-1} concentrations of the test compounds in 3.4 mL phosphate buffer were added to the H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of H_2O_2 scavenging was calculated using equation 1.[34] The results were expressed as EC_{50}.

Acetylcholinesterase inhibitory activity

The enzymatic inhibition was measured using the method described by Canto *et al.*[11] with modifications. Briefly were mixed: 90 µL of Tris-HCl buffer (of concentration 50 mmol L^{-1}, pH 8.0), 30 µL of methanolic solution of the compound (0.1 mg mL^{-1}) and 15 µL of an AChE solution (0.25 U mL^{-1}). The AChE solution was prepared by dissolving 0.1% of bovine serum albumin in 50 mmol L^{-1} of Tris-HCl pH 8 buffer. The mixture was incubated for 15 min. Then, 25 µL of an acetylthiocholine iodide solution (15 mmol in water) and 140 µL of Ellman's reagent (3 mmol L^{-1} in Tris-HCl pH 8.0 buffer containing 0.1 mol L^{-1} NaCl and 0.02 mol L^{-1} $MgCl_2$) were added, and the final mixture was incubated for another 30 min at 28 °C. The absorbance of the mixture was measured at 405 nm. The same solvent in which the sample was dissolved, considered to have 100% AChE activity, was used as negative control. The inhibition (%) was calculated as follows, in which A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance without the sample:

$$I(\%) = \left(100 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad (2)$$

The tests were performed in triplicate, and a blank containing Tris-HCl buffer was used instead of the enzyme solution. The concentration required to inhibit enzyme activity by 50%, IC_{50}, was determined from the graph-plotted concentration *versus* absorbance. Remynil® (0.1 mg mL^{-1}) was used as the positive control, which contains 50% of galantamine.

Statistical analysis

All experiments were performed three times and the

mean results were calculated. Data was expressed as mean ± SD. Linear regression analysis was used to calculate EC_{50} (for antioxidant assays) or IC_{50} (for acetylcholinesterase inhibitory activity) for each compound.

Quantum studies

The molecular representation sketch of the reference compound was plotted using ChemBioOffice 2010 software. All quantum chemical calculations were performed using the MM2 methodology.

Results and Discussion

The synthesis of thiosemicarbazones **2a**-**q** was performed in two stages, according to Scheme 1. First, the synthesis of thiosemicarbazide **1** was performed by the reaction between 1-isothiocyanato-4-methylbenzene and hydrazine hydrate in isopropanol.[33] Compound **1** was used in subsequent reactions with different aldehydes using acetic acid as a catalyst in a solvent mixture of water/ethanol[26] to yield thiosemicarbazones **2a**-**q**. Thiosemicarbazones **2g**, **2i**, **2k** and **2l** have not been reported in the literature. In general, the synthesis of all compounds showed similar yields (ranging 60-97%).

All synthesized compounds were unequivocally characterized by their melting points, IR, ^{1}H NMR, and ^{13}C NMR, and the structures were confirmed by HRMS. In the compound's ^{1}H NMR spectra, a singlet signal at the 2.30-2.39 region is attributed to a CH_3 moiety, and aromatic hydrogens appear at 6.5-8.5 ppm, depending on the substituent in the aromatic (Ar) group. The iminic hydrogen is assigned as a singlet at 7-8 ppm and the N–H chemical shifts appear as two singlets around 9-12 ppm. The ^{13}C NMR spectra of the compounds showed signals between 100.0 and 160.0 ppm due to the resonance of aryl and unsaturated carbons. The signals at the region between 170 and 180 ppm, attributed to the carbon resonance of the C=S group, are consistent with the expected structures.

Antioxidant activity

The evaluation of a compound's antioxidant activity should not be solely based on a single method.[10] Several *in vitro* antioxidant tests are reported in the literature, and in this study, the antioxidant activity of the compounds **2a**-**q** was analyzed using two *in vitro* assays, which were correlated with the quantum assays.

Nitric oxide (NO) has a role in the regulation of various physiological processes and is an important chemical mediator. Moreover, the NO pathway is involved in various types of disorders, as Alzheimer's disease.[36]

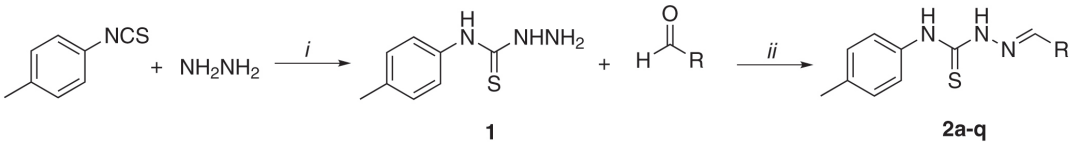
Hydrogen peroxide (H_2O_2) is rapidly decomposed into oxygen and water, and this can produce hydroxyl radicals ($OH^{\bullet}$) that initiate lipid peroxidation and cause DNA damage in the body, thus becoming a target for the research of new compounds.[10]

The results of nitric oxide scavenging activity and hydrogen peroxide scavenging activity of compounds **2a**-**q** are shown in Table 1. All analyzed compounds were more active than the standard (ascorbic acid). Compound **2j** was the most active and compound **2o** the less active from this series. The results of both experimental activities are linearly correlated ($y = 0.96810x + 2.07336$; $R^2 = 0.99995$), indicating consistent results between both tests.

Acetylcholinesterase inhibitory activity

All studied compounds were evaluated as AChE inhibitors. The concentration of test compounds that inhibited substrate hydrolysis by 50% (IC_{50}, Table 1) was determined by plotting the inhibition percentage against the sample solution concentrations.

Thiosemicarbazones **2a**-**2q** showed a high percentage of inhibition of the enzyme acetylcholinesterase, being as active as the galantamine standard (active drug used for the treatment of Alzheimer's disease, Remynil®). The result obtained for the standard is in accordance with literature data.[11] The most active compounds in the series were **2c**,



2a, R= Ph
2b, R= 4-CH_3-Ph
2c, R= 4-NO_2-Ph
2d, R= 4-Cl-Ph
2e, R= 4-OCH_3-Ph
2f, R= 3,4-OCH_2O-Ph
2g, R= 4-Ph-Ph
2h, R= 3-OCH_3,4-OH-Ph
2i, R= 3,4,5-triOCH_3-Ph
2j, R= 1-naphthyl
2k, R= 2-naphthyl
2l, R= 3,4-diCl-Ph
2m, R= 2,5-diOCH_3-Ph
2n, R= 3,4-diOCH_3-Ph
2o, R= 2-NO_2-Ph
2p, R= 5-(2-Cl,5-CF_3)-Ph-furan-2yl
2q, R= 4-N$(CH_3)_2$-Ph

Scheme 1. Synthesis of thiosemicarbazide **1** and thiosemicarbazones **2a**-**q**. (*i*) Isopropanol, r.t.; (*ii*) ethanol/water, acetic acid, reflux.

Table 1. Antioxidant activity and acetylcholinesterase inhibitory assays of compounds **2a**-**2q**

Compound	Nitric oxide (NO) scavenging activity EC_{50} / (μmol L^{-1})	Hydrogen peroxide (H_2O_2) scavenging activity EC_{50} / (μmol L^{-1})	AChE inhibition IC_{50} / (μmol L^{-1})
2a	8.856	10.654	6.12
2b	8.758	10.556	6.36
2c	9.681	11.483	1.98
2d	8.714	10.512	9.44
2e	8.788	10.585	8.01
2f	8.649	10.450	10.33
2g	8.784	10.583	4.66
2h	8.862	10.661	9.86
2i	8.778	10.571	8.94
2j	8.631	10.429	2.26
2k	8.753	10.551	4.45
2l	8.646	10.448	4.16
2m	8.645	10.443	7.28
2n	8.855	10.653	8.56
2o	10.203	12.006	2.04
2p	8.632	10.431	6.54
2q	8.726	10.528	14.26
Ascorbic acid	14.75	16.35	–
Galantamine	–	–	17.05

2o and **2j**. These compounds have a NO_2 group (**2c**, **2o**) or a naphthyl group (**2j**) in their structures.

By analyzing the data obtained for the acetylcholinesterase enzyme inhibition assay it is possible to perceive a strong correlation between the IC_{50} value and the substituent groups present on the ring. The two compounds that presented lower IC_{50} values, therefore more active, have in their structure groups that withdraw electrons, especially the nitro group for compounds **2c** and **2o**. Similarly, the compounds for which the lowest enzymatic inhibition activity, **2q** and **2f**, were observed, present groups capable of increasing the electronic density of the aromatic ring, by donor effect.

Compounds **2j** and **2k**, which have a naphthyl substituent, can be highlighted since the results can be considered to be good for both enzymatic inhibition and antioxidant assays. As well as, a thiosemicarbazone **2l**, which have a 3,4-diCl-Ph substituent, also showed a good ratio for the biological assay. Clearly, the ability to act as donors or as electron withdrawals contributes to the good results mentioned.

Quantum studies

Quantum studies was employed to analyze thiosemicarbazones **2a**-**q**. The HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) energy values were calculated and are shown in Table 2. The HOMO and LUMO band gap (energy difference between HOMO and LUMO) was determined for the compounds, as illustrated in Figure 1. Thus, it was possible to search for relations between the *in vitro* tests results and the theoretical data.

E_{HOMO} was a better indicator of antioxidant activity than E_{LUMO}. In general, it is possible to relate HOMO energy values and scavenging activities. Compound **2a**, however, did not show a direct relationship between E_{HOMO} and antioxidant activity, presenting a high HOMO energy value and a low NO and H_2O_2 scavenging activity when compared to the other compounds. This case aside, E_{HOMO} was considered a good indicator of antioxidant activity for the tested series in this study.

E_{LUMO} was a better indicator than E_{HOMO} regarding acetylcholinesterase inhibitory activity.

Other properties can also be deducted from the analysis of HOMO and LUMO energy values, such as a compound's ionization potential (IP), electron affinity (EA), electronegativity (μ), hardness (η) and softness (S).[37]

The ionization potential (IP) of thiosemicarbazones **2a**-**q** was obtained using equation 3.[37] Therefore, the lower the energy required to remove an electron, higher is the

Table 2. Energy values of HOMO, LUMO and band gap of thiosemicarbazones **2a**-**q**

Compound	E_{HOMO} / eV	E_{LUMO} / eV	Band gap ($E_{HOMO} - E_{LUMO}$)
2a	–6.228	–2.514	3.714
2b	–6.219	–2.278	3.941
2c	–6.874	–5.453	1.421
2d	–6.187	–1.771	4.416
2e	–6.240	–1.874	4.366
2f	–6.142	–1.298	4.844
2g	–6.238	–3.718	2.520
2h	–6.293	–1.747	4.546
2i	–6.233	–1.837	4.396
2j	–6.099	–4.284	1.815
2k	–6.215	–3.824	2.391
2l	–6.140	–1.803	4.337
2m	–6.138	–2.094	4.044
2n	–6.282	–1.858	4.424
2o	–7.245	–5.285	1.960
2p	–6.129	–2.172	3.957
2q	–6.197	–0.539	5.658
Ascorbic acid	–10.474	–1.303	9.171
Galantamine	–8.968	0.703	8.265

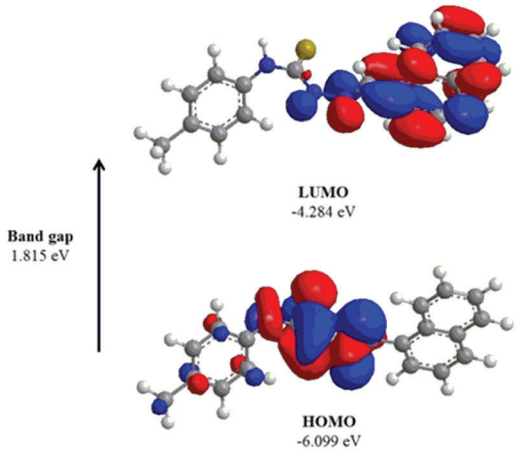


Figure 1. Band gap energy for compound **2j**.

compound's antioxidant activity, or, in other words, the higher the E_{HOMO}, smaller is the IP.

$$IP = -E_{HOMO} \quad (3)$$

The EA of thiosemicarbazones **2a**-**q** was obtained using equation 4.[37] High EA values represent that an electron can be easily added to the species, being directly related to the compound's antioxidant capability.

$$EA = -E_{LUMO} \quad (4)$$

Electronegativity (μ) of thiosemicarbazones **2a**-**q** was obtained using equation 5.[37] Thus, a compound with a lower electronegativity is expected to have a higher antioxidant activity.

$$\mu = -\frac{1}{2}(E_{HOMO} + E_{LUMO}) \quad (5)$$

The interaction of valence shell electrons with the nucleus and intervening filled shells also affects the polarizability of the valence shell electrons. Polarizability can be described in terms of hardness and softness. A relatively large atom or ion with a small effective nuclear charge is relatively easily distorted (polarized) by an external charge and is called soft.[38] A more compact electron distribution resulting from a higher net nuclear charge and less effective screening is called hard. The η and S of thiosemicarbazones **2a**-**q** were obtained using equations 6 and 7.[37]

$$\eta = -\frac{1}{2}(E_{HOMO} - E_{LUMO}) \quad (6)$$

$$S = -\frac{2}{(E_{HOMO} - E_{LUMO})} \quad (7)$$

The values obtained for IP, EA, μ, η and S can be seen in Table 3.

The chemical properties that were theoretically calculated from the LUMO values did not present a good correlation with the antioxidant activities, which was already expected, since the E_{LUMO} values were not good indicators. However, a linear correlation was observed between the IP and the nitric oxide (NO) scavenging activity ($y = 1.3981x + 0.0719$; $R^2 = 0.99699$) and between the IP and the hydrogen peroxide (H_2O_2) scavenging activity ($y = 1.40210x + 1.84593$; $R^2 = 0.99705$).

The EA presented a better correlation with the results of acetylcholinesterase inhibition assays, but it was not as linear as the antioxidant activity. In general, the higher the electron affinity value, the higher the inhibition of acetylcholinesterase. Furthermore, a relation with hardness and softness can be found considering only the best and the worst results of the enzymatic inhibition test. In this study, it was possible to observe that the softer compound was the one that presented the better results (**2c**), and the harder, the worse results (**2q**). The correlations found involving quantum studies, in relation to biological activities evaluated at this work, are of a qualitative nature.

Table 3. Chemical properties of thiosemicarbazones **2a**-**q**

Compound	Ionization potential / eV	Electron affinity / eV	Hardness (η)	Softness (S)	Electronegativity (μ)
2a	6.228	2.514	1.857	0.5385	4.371
2b	6.219	2.278	1.970	0.5075	4.248
2c	6.874	5.453	0.7105	1.407	6.163
2d	6.187	1.771	2.208	0.4529	3.979
2e	6.240	1.874	2.183	0.4581	4.057
2f	6.142	1.298	2.422	0.4129	3.720
2g	6.238	3.718	1.260	0.7936	4.978
2h	6.293	1.747	2.273	0.4399	4.020
2i	6.233	1.837	2.198	0.4549	4.035
2j	6.099	4.284	0.9075	1.102	5.191
2k	6.215	3.824	1.195	0.8365	5.019
2l	6.140	1.803	2.168	0.4611	3.971
2m	6.138	2.094	2.022	0.4945	4.116
2n	6.282	1.858	2.212	0.4521	4.070
2o	7.245	5.285	0.980	1.020	6.265
2p	6.129	2.172	1.978	0.5054	4.150
2q	6.197	0.539	2.829	0.3535	3.368
Ascorbic acid	10.47	1.303	4.585	0.2181	5.888
Galantamine	8.968	–0.703	4.132	0.2420	4.835

For the antioxidant activity, the bulky groups of compounds **2j** and **2p** (1-naphthyl and 5-(2-Cl-5-CF_3)-Ph-furan-2yl, respectively) appear to be important for the assays performed in this study. In addition, thiosemicarbazones **2c** and **2o** presented the worst results of the series; both compounds have a nitro group (NO_2) in their structure, concluding that this electron-withdrawing group decreases antioxidant activity.

Regarding the inhibitory activity of acetylcholinesterase, the nitro group (NO_2) appeared to be important for a higher rate of inhibition, a fact that differs from the antioxidant activity. However, the 1-naphthyl group presented good results for both activities, thus thiosemicarbazone **2j** is a potential therapeutic agent for AD treatment, since it has multiple actions, acting as an efficient inhibitor of acetylcholinesterase and as a potent antioxidant.

Conclusions

In this study, the synthesis of seventeen thiosemicarbazones was performed and the compounds were analyzed for AChE inhibition potency and antioxidant activity. All compounds showed excellent activity as inhibitors of AChE, being more active than the standard drug. Nitric oxide (NO) scavenging activity and hydrogen peroxide (H_2O_2) scavenging activity assays were employed and the results showed good antioxidant activity. Compound **2j** showed multiple action, a property that makes it a potential therapeutic agent for AD. In addition, quantum studies showed that E_{HOMO} values were a better indicator of antioxidant activity than E_{LUMO} values, while E_{LUMO} was a better indicator of AChE inhibition.

Supplementary Information

Supplementary information with analytical data (^{1}H and ^{13}C NMR and HOMO and LUMO of the surface) is available free of charge at http://jbcs.sbq.org.br as a PDF file.

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