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DESIGN, MICROWAVE-ASSISTED SYNTHESIS, BIOLOGICAL EVALUATION, MOLECULAR DOCKING, AND ADME STUDIES OF PYRROLE-BASED HYDRAZIDE-HYDRAZONES AS POTENTIAL ANTIOXIDANT AGENTS

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In this study, one novel *N*-pyrrolyl carboxylic acid (**3**), the corresponding *N*-pyrrolyl hydrazide (**5**), and four new hydrazide-hydrazones (**5a-d**) bearing electron donating moieties were designed, synthesized, and fully elucidated by ^{1}H NMR, FT-IR, and HRMS. The hydrazide-hydrazones were produced in five steps, which were optimized by applying microwave heating. The microwave-assisted synthesis significantly decreased the reaction times and increased the yields of the title molecules. In addition, all novel compounds were assessed for their radical scavenging properties by employing DPPH and ABTS assays. The most promising agent was obtained after condensation of the title hydrazide (**5**) with a 3,5-dimetoxy-4-hydroxybenzaldehyde (**5d**). The latter compound showed better antioxidant properties than Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and could serve as a prominent lead structure for future optimization as an antioxidant agent. A possible binding conformation of **5d** in the active site of NADPH oxidase was also identified through molecular docking simulations. Analysis of the major interactions showed the importance of the hydroxyl moiety for the antioxidant activity. Finally, the virtual calculations of the ADME properties of the synthesized compounds displayed good drug-like properties. Overall, an optimized synthetic protocol through MW irradiation was employed. The newly synthesized ethyl (*E*)-5-(4-bromophenyl)-1-(1-(2-(4-hydroxy-3,5-dimethoxybenzylidene)hydrazineyl)-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl)-2-methyl-1*H*-pyrrole-3-carboxylate (**5d**) was found to possess the most prominent radical-scavenging capacity, which identifies it as a promising lead compound for the development of novel antioxidants.

Keywords: microwave-assisted synthesis; pyrrole; hydrazide-hydrazones; antioxidants; molecular docking

ДИЗАЈН, МИКРОБРАНОВО ПОМОГНАТА СИНТЕЗА, БИОЛОШКА ЕВАЛУАЦИЈА, МОЛЕКУЛСКО ПРИПОЈУВАЊЕ И ADME СТУДИИ НА ХИДРАЗИД-ХИДРАЗОНИ НА ПИРОЛСКА ОСНОВА КАКО ПОТЕНЦИЈАЛНИ АНТИОКСИДАЦИСКИ СРЕДСТВА

Во ова истражување беше дизајнирана, синтетизирана и целосно потврдена со ^{1}H NMR, FT-IR и HRMS една нова *N*-пиролил карбоксилна киселина (**3**), како и соодветниот пиролил хидразид (**5**) и четири нови хидразид-хидразони (**5a-d**) што во структурата имаат електронски донорски делови. Хидразид-хидразоните беа добиени во пет чекори што беа оптимизирани со примена на микробраново загревање. Микробрановo помогнатата синтеза значајно ги намали времињата на реакција, а ги зголеми приносите на наведените соединенија. Покрај тоа, за сите соединенија беше направена процена на нивната способност за отстранување електрони со употреба на DPPH и ABTS анализи. Се покажа дека најмногу ветува насловниот хидразид (**5**) со 3,5-диметокси-4-хидроксибензалдехид (**5d**). Ова соединение покажа подобри антиоксидациски својства од Trolox (6-хидрокси-2,5,7,8-тетраметилхроман-2-карбоксилна киселина) и може да служи како важна примерна структура за идно оптимизирање на антиоксидациски средства. Исто така беше идентификувана конфорамција на активниот центар за сврзување на **5d** со оксидазата на NADPH

преку симулации на молекулско припојување. Анализата на главните интеракции ја покажа важноста на хидроксилниот дел за антиоксидацискатa активност. Конечно, виртуелните пресметки на ADME–својствата на синтетизираните соединенија покажаа добри особини својствени за лекови. Се покажа дека новосинтетизираниот етил (*E*)-5-(4-бромофенил)-1-(1-(2-(4-хидрокси-3,5-диметоксибензилиден)хиразинил)-3-(1*H*-индол-3-ил)-1-оксопропан-2-ил)-2-метил-1*H*-пирол-3-карбоксилат (**5d**) има најголем капацитет за отстранување на радикали, што го прави потенцијално ветувачко соединение за развој на нови антиоксиданси.

Клучни зборови: микробраново помогната синтеза; пирол; хидразид-хидразони; антиоксданси; молекулско припојување

1. INTRODUCTION

The balance between the antioxidant systems of the body and the generated free radicals is essential. Elevated concentrations of reactive oxygen species (ROS) formed within mitochondria could break the balance and initiate oxidative stress that damages living cells.[1] The ROS radicals consist of superoxide ($O_2^{\cdot}$), peroxyl (ROO·), alkoxyl (RO·), hydroxyl (HO·), and nitric oxide (NO·) oxygen centered free radicals. Additionally, the production of ROS has been related to aging, obesity, depression, cancer, cataracts, and most of the neurodegenerative diseases.[2,3] Compounds with antioxidant properties have the potential to prevent or to delay the aforementioned diseases.[4]

Molecules comprising a hydrazide-hydrazone moiety are an essential class of organic compounds, formed by the reaction of hydrazide and ketones/aldehydes. The pharmacological profile of the latter functional group is broad, and it includes antimicrobial, antiseptic, antidepressant, antitubercular, antifungal, anti-inflammatory, antiviral, and antiprotozoal effects.[5] Literature data describing the antioxidant properties of hydrazide-hydrazone-based compounds are also published.[6] Thus, an increasing number of medicinal chemists are interested in the design and synthesis of various compounds comprising a hydrazide-hydrazone moiety.

Recently, the utilization of microwave-assisted synthesis has been growing exponentially because of the significant reduction in the reaction time.[7] It has been reported that the the main advantage of MW (microwave) synthesis over conventional heating is the direct transfer of heat. Therefore, the reactions are completed in minutes compared to conventional conditions.[8] Furthermore, higher yields, rapid cooling potential, higher product purities, minor pollution of the environment, and easier work-up practices have been discussed.[9,10]

Therefore, the aim was to implement both conventional and microwave-assisted synthesis of one *N*-pyrrolylcarboxylic acid (**3**), the corresponding hydrazide (**5**), and four novel hydrazide-hydrazones (**5a-d**). The antioxidant properties of the latter were assessed with DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging tests. Further molecular docking simulations in the active site of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase were carried out. Finally, in silico ADME (absorption, distribution, metabolism, and excretion) parameters of all title compounds were determined to evaluate their pharmacokinetic properties.

2. MATERIALS AND METHODS

2.1. *Chemistry*

Available reactants and solvents were obtained from commercial suppliers and applied without further purification. The microwave-assisted synthesis was carried out by utilizing a FlexiWave Milestone Lab Microwave reactor (equipped with a fibre optic sensor). Thin layer chromatography (TLC) was used for monitoring the reactions. The TLC characteristics were measured on aluminum sheets of silica gel 60 F254, Merck 1.05554 at ambient temperature, employing a mobile phase – chloroform:ethanol = 10:0.6. The melting points were determined by a Kruss M5000. The UV/Visible scans were conducted with a Jenway 6715 Spectrophotometer. IR spectra (4000–400 cm^{-1} range) were recorded on a Nicolet iS10 FT-IR spectrometer with the Smart iTR adapter (Thermo Fisher Scientific, USA). ^1H spectra were registered on a Bruker Avance II+ 600 (Biospin GmbH, Rheinstetten, Germany) as δ (ppm) relative to TMS (Tetramethylsilane) as an internal standard, and the coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (δ) were expressed in parts per million (ppm) relative to deuterated chloroform ($CDCl_3$) as a solvent. The mass spectra were obtained with a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface (Agilent Technologies, USA).

General procedure for the synthesis of 1,4-dicarbonyl compounds (2)

The synthesis of the dicarbonyl compound (**2**) was accomplished according to a recently reported procedure by Bijev et al.[11]

General procedure for the synthesis of *N*-pyrrolylcarboxylic acid (3)

One mol ethyl 2-acetyl-4-(4-bromophenyl)-4-oxobutanoate (**2**) and 1.2 mol of tryptophan were dissolved in 60 ml of glacial acetic acid. The mixture was heated by conventional and microwave-assisted techniques. The conventional heating required 36 h of reflux conditions, while the microwave assisted synthesis completed the reaction within 55 min. The microwave reactor was set to 700 W and 75 °C, and the stirring capacity was 70 %. Subsequently, the mixture was cooled at ambient temperature and poured into ice water. The precipitate was dissolved in CH_3Cl, worked up with 5 % NaOH and 5 % HCl, and washed several times with water. The final product was dried and re-crystallized from ethanol.

*2-[5-(p-Bromophenyl)-3-ethoxycarbonyl-2-methyl-1H-pyrrol-1-yl]-3-(1H-indol-3-yl)propionic acid **(3)***

89 % yield; 174–174.3 °C melting point; TLC 0.30 (10:0.2); IR ν_{max}: 3350 cm^{-1} (OH); 1630 cm^{-1} (C=O); 740, 710 cm^{-1} (*p*-C_6H_4); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 12.70 (1H, s, O<u>H</u>), 10.73 (1H, s, N<u>H</u> (tryptophan)), 7.76–7.78 (2H, t, *J* = 7.55 Hz, H-3`, H-5`), 7.55 (2H, t, *J* = 7.50 Hz, H-2`, H-6`), 7.33 (2H, t, *J* = 8.1 Hz, H-5, H-6), 7.18 (1H, s, H-2), 7.06 (1H, d, H-7 (tryp.)), 6.78 (1H, s, H-4), 5.05 (1H, t, *J* = 7.90 Hz, CH_2-C<u>H</u>-(N)CO), 4.22 (1H, s, H-2 (tryp.)), 3.45 (4H, m, C<u>H$_2$</u>-CH_3), 3.05 (2H, d, C<u>H$_2$</u>-CH), 2.47 (3H, s, C<u>H$_3$</u>), 1.30 (3H, t, *J* = 7.30 Hz, CH_2-C<u>H$_3$</u>); *m/z* (FTMS+pESI) 495.74.

General procedure for the synthesis of ethyl ester of *N*-pyrrolylcarboxylic acid (4)

Initially, $SOCl_2$ (0.2 mol) was added dropwise to 200 ml of absolute ethanol at 0 °C. The reaction mixture was stirred for 30 min, and thereafter, 0.05 mol of compound **2** was added. The synthesis proceeded by both conventional and microwave heating. The synthesis of **4** by the conventional method required 24 h of reflux conditions. The microwave settings were set at 75 °C and power of 700 W. The latter heating needed 40 min for the completion of the reaction (TLC control). The solvent was evaporated and washed with 5 % Na_2CO_3. The obtained ester was incorporated in the next synthetic phase without further purification and isolation.

General procedure for the synthesis of hydrazide of the *N*-pyrrolylcarboxylic acid (5)

A hydrazide of the *N*-pyrrolylcarboxylic acid **(5)** was produced by direct hydrazinolysis of the obtained ethyl ester (**4**). 0.04 mol of the latter ester and 0.2 mol hydrazine hydrate (100 %) were dissolved in 55 ml of absolute ethanol. The reaction was carried out by conventional and microwave heating. The classic reflux condition required prolonged heating of 168 h (7 days), while the microwave-assisted hydrazinolysis of **4** was completed in 2 h (700 W, 80 °C). The final product was isolated after cooling the reaction mixture and washed with ethanol.

*Ethyl 5-(4-bromophenyl)-1-(1-hydrazineyl-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **(5)***

87 % yield; 214.4–214.6 °C melting point; TLC 0.30 (10:0.4); IR ν_{max}: 3303–3156 cm^{-1} (NH); 2955 cm^{-1} (NH_2); 1725 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 10.70 (1H, s, N<u>H</u> (tryptophan)), 9.4 (1H, s, CO-N<u>H</u>), 7.25 (2H, t, *J* = 8.10 Hz, H-3`, H-5`), 7.05 (2H, t, *J* = 7.15 Hz, H-2`, H-6`), 6.74 (2H, m, H-5, H-6 (tryp.)), 6.52 (1H, d, *J* = 6.40 Hz, H-7 (tryp.)), 6.15 (1H, s, H-4), 4.9 (1H, t, *J* = 4.10 Hz, CH_2-C<u>H</u>-(N)CO), 4.45 (1H, s, H-2 (tryp.)), 4.31 (2H, s, NH-<u>NH$_2$</u>), 4.16 (4H, m, C<u>H$_2$</u>-CH_3), 3.5 (2H, d, *J* = 4.8, Hz, C<u>H$_2$</u>-CH), 3.3 (3H, s, C<u>H$_3$</u>), 1.25 (3H, t, *J* = 7.10 Hz, CH_2-C<u>H$_3$</u>); *m/z* (FTMS+pESI) 508.47.

General procedure for the synthesis of hydrazide-hydrazones (5a-d)

The hydrazide-hydrazones were synthesized after reacting equimolar quantities (2 mmol) of the hydrazide (**5**) and 2 mmol concentration of the corresponding carbonyl partners: 4-methoxybenzaldehyde (**a**), 3,5-dimethoxybenzaldehyde (**b**), 2,4-dimetoxybenzaldehyde (**c**), and 3,5-dimethoxy-4-hydroxybenzaldehyde (**d**). 6 ml of glacial acetic acid was applied as a solvent. When the conventional heating was utilized, the condensations were completed in 30–50 min. The microwave heating reduced the reaction time to 30 s and increased the yields. All of the hydrazide-hydrazones participated when cold water was added to the reaction mixture. Recrystallization in ethanol was carried out.

*Ethyl (E)-1-(3-(1H-indol-3-yl)-1-(2-(4-methoxybenzylidene)hydrazineyl)-1-oxopropan-2-yl)-5-(4-bromophenyl)-2-methyl-1H-pyrrole-3-carboxylate (**5a**)*

92 % yield; 194–194.7 °C melting point; TLC 0.49 (10:0.4); IR ν_{max}: 3010 cm^{-1} (NH), 1680 cm^{-1} (C=O), 1600 cm^{-1} (C=N); ^{1}H NMR: ($CDCl_3$, 600 MHz) δ 10.79 (1H, s, NH (tryptophan)), 8.47 (1H, s, CO-NH), 8.21 (1H, s, N-CH), 7.80 (1H, d, *J* = 6.60 Hz, H-7 (tryp.)), 7.66 (2H, t, H-2``, H-6`` (aldehyde)), 7.45 (2H, t, *J* = 8.00 Hz, H-3`, H-5`), 7.30 (2H, t, *J* = 7.20 Hz, H-2`, H-6`), 7.21 (2H, t, H-3``, H-5`` (ald.)), 7.15 (2H, m, H-5, H-6 (tryp.)), 6.95 (1H, s, H-4), 6.20 (1H, s, H-2 (tryp.)), 5.10 (1H, t, *J* = 6.50 Hz, CH_2-CH-(N)CO), 4.29 (2H, q, CH_2-CH_3), 3.81 (2H, d, *J* = 6.90 Hz, CH_2-CH), 2.83 (3H, s, CH_3), 1.81 (3H, s, -OCH_3), 1.34 (3H, t, *J* = 8.10 Hz, CH_2-CH_3); *m/z* (FTMS+pESI) 627.52.

*Ethyl (E)-5-(4-bromophenyl)-1-(1-(2-(2,4-dimethoxybenzylidene)hydrazineyl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **(5b)***

91 % yield, 139.9–140.6 °C melting point; TLC 0.58 (10:0.4); IR ν_{max}: 2931 cm^{-1} (NH), 1670 cm^{-1} (C=O), 1596 cm^{-1} (C=N); ^{1}H NMR: ($CDCl_3$, 600 MHz) δ 10.18 (1H, s, NH (tryptophan)), 8.78 (1H, s, CO-NH), 8.37 (1H, s, N-CH), 7.76 (1H, d, H-7 (tryp.)), 7.50 (1H, d, *J* = 7.10 Hz, H``-6 (ald.)), 7.25 (2H, m, H-5, H-6 (tryp.)), 7.06 (2H, t, *J* = 8.10 Hz, H-3`, H-5`), 6.78 (1H, s, H-4), 6.67 (2H, t, *J* = 8.30 Hz, H-2`, H-6`), 6.47 (2H, t, *J* = 7.80 Hz, H-5``, H-6`` (ald.)), 5.45 (1H, s, H-2 (tryp.)), 5.10 (1H, t, *J* = 6.10 Hz, CH-(N)CO), 4.35 (4H, m, CH_2-CH_3), 3.81 (3H, s, CH_3), 3.68 (2H, d, CH_2-CH), 2.35 (3H, s, -OCH_3 (ald.)), 2.42 (3H, s, -OCH_3 (ald.)), 1.34 (3H, t, *J* = 7.85 Hz, CH_2-CH_3); *m/z* (FTMS+pESI) 657.14.

*Ethyl (E)-5-(4-bromophenyl)-1-(1-(2-(2,3-dimethoxybenzylidene)hydrazineyl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **(5c)***

94 % yield; 232.9–233.2 °C melting point; TLC 0.52 (10:0.4); IR ν_{max}: 3450 cm^{-1} (NH), 1689 cm^{-1} (C=O), 1664 cm^{-1} (C=N); ^{1}H NMR: ($CDCl_3$, 600 MHz) δ 10.18 (1H, s, NH (tryptophan)), 8.66 (1H, s, CO-NH), 8.40 (1H, d, H-7 (tryp.)), 8.23 (1H, s, N-CH), 7.62 (1H, d, *J* = 7.20 Hz, H-6`` (ald.)), 7.36 (2H, t, *J* = 8.35 Hz, H-3`, H-5`), 7.29 (2H, t, *J* = 8.15 Hz, H-2`, H-6`), 7.20 (2H, m, H-5, H-6 (tryp.)), 7.13 (1H, d, *J* = 6.50 Hz, H-4`` (ald.)), 6.99 (1H, t, *J* = 7.25 Hz, H-5`` (ald.)), 6.65 (1H, s, H-4), 5.90 (1H, s, H-2 (tryp.)), 5.15 (1H, t, *J* = 4.10 Hz, CH-(N)CO), 4.30 (4H, m, CH_2-CH_3), 3.68 (2H, d, CH_2-CH), 2.91 (3H, s, CH_3), 1.71 (3H, s, -OCH_3 (ald.)), 1.61 (3H, s, -OCH_3 (ald.)), 1.35 (3H, t, *J* = 7.55 Hz, CH_2-CH_3); *m/z* (FTMS+pESI) 657.55.

*Ethyl (E)-5-(4-bromophenyl)-1-(1-(2-(4-hydroxy-3,5-dimethoxybenzylidene)hydrazineyl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2-methyl-1H-pyrrole-3-carboxylate **(5d)***

88 % yield; 170.8–171.4 °C melting point; TLC 0.20/(10:0.4); IR ν_{max}: 3390 cm^{-1} (NH), 1669 cm^{-1} (C=O), 1588 cm^{-1} (C=N); ^{1}H NMR: ($CDCl_3$, 600 MHz) δ 10.79 (1H, s, NH (tryptophan)), 8.55 (1H, s, CO-NH), 8.41 (1H, s, N-CH), 8.20 (1H, s, OH), 7.86 (1H, d, H-7 (tryp.)), 7.55 (2H, t, *J* = 8.15 Hz, H-3`, H-5`), 7.33 (2H, t, *J* = 7.80 Hz, H-2`, H-6`), 7.08 (2H, m, H-5, H-6 (tryp.)), 6.93 (2H, s, H-2``, H-6`` (ald.)), 6.80 (1H, s, H-4), 6.10 (1H, s, H-2 (tryp.)), 5.10 (1H, t, *J* = 7.50 Hz, CH-(N)CO), 4.25 (4H, m, CH_2-CH_3), 3.65 (2H, d, CH_2-CH), 2.85 (3H, s, CH_3), 1.80 (3H, s, -OCH_3), 1.34 (3H, t, *J* = 7.50 Hz, CH_2-CH_3); *m/z* (FTMS+pESI) 673.84.

DPPH assay

Initially, the radical scavenging activities of **3**, **5**, and **5a-d** were initially determined with the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. DPPH is a stable free-radical molecule regularly utilized for antioxidant assays. A methanol solution of DPPH has an intense violet color with UV absorption at 515 nm. In the presence of antioxidants or free radical species, the DPPH solution is decolorized and the inhibition capacities are monitored by the decrease in the absorption. In the current study, the scavenging rate of the DPPH radical was carried out by the widely employed classical protocol of Brand-Williams et al.[12] Briefly, five different concentrations ranging from 31 to 250 μM of each examined compound in methanol were obtained (1 ml), followed by the addition of 1 ml of the methanol solution of DPPH (1 mmol/l). Each reaction mixture was incubated in the dark for 30 min. The decrease in the absorbance was measured at 517 nm. Three measurements were carried out for each sample. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard. The percentage inhibition of the tested samples was calculated by the following formula (1):

$$DPPH_{scavenging\ activity} = Abs_{control} - Abs_{sample} / Abs_{control} \times 100\% \quad (1)$$

where $Abs_{control}$ is the absorbance of the DPPH radical in methanol and Abs_{sample} is the absorbance of the DPPH radical solution mixed with the sample.

ABTS assay

The ABTS radical scavenging activities of the title compounds were measured according to a modified method of Arnao et al.[13] The analyses were carried out in methanol at ambient temperature, and the absorbance was measured at $\lambda = 734$ nm. The radical cation ($ABTS^{+\bullet}$) was generated by mixing 7 mmol/l solution of ABTS and 2.4 mmol/l solution of potassium persulphate, which were allowed to react for 14 h in the dark at room temperature. The working solutions comprised 2 ml of the stock solution diluted in 50 ml of methanol with an absorbance of 0.302 ± 0.04 units at 517 nm. 1 ml of the ABTS working solution was allowed to react with three different concentrations of the pyrrole derivatives (31, 125, 250 μM) for 10 min, with a subsequent absorbance determination. The inhibition percentage was calculated by applying the same formula as the DPPH assay.

Molecular docking

The active conformations of the top scored ligands were detected with molecular docking. The simulations were carried out with Glide (Schrödinger Release 2021-3: Glide, Schrödinger, LLC, New York, NY, 2021) in the active site of NADPH oxidase (PDB:**2CDU**). The crystal structure was retrieved from the Protein Data Bank (PDB) (https://www.rcsb.org) with a co-crystallized ligand and resolution of 1.80 Å. Resolutions under 2 Å demonstrate higher reliability when compared to crystallographic structures resolved with resolutions over 2 Å. The Protein Preparation module in Schrödinger was employed for the preparation steps of the receptor. The Receptor Grid Generator in Maestro was utilized for the determination of the grid space, which was centered on the co-crystallized ligand. The title compounds were drawn in ChemDraw (PerkinElmer Informatics) and converted to the corresponding 3D structures with the LigPrep module in Maestro. Utilizing the former module, the addition of hydrogen atoms, bond order assignment, and energy minimization with OPLS force field were carried out. The active waters were retained. Three different docking precisions were employed in Glide – High throughput virtual screening (HTVS), Standard precision (SP), and Extra precision (XP). For the current study, the XP mode was utilized, with Van der Waals radii cutoff of 0.15 and scaling factor of 0.80. Epik state penalties to the docking scores were included. Torsional constraints were not used. The major interactions between compound **5d** and the active site of the receptor were visualized with the XP Visualizer Maestro (Schrödinger Release 2021-3: Maestro, Schrödinger, LLC, New York, NY, 2021).

Prediction of ADME properties

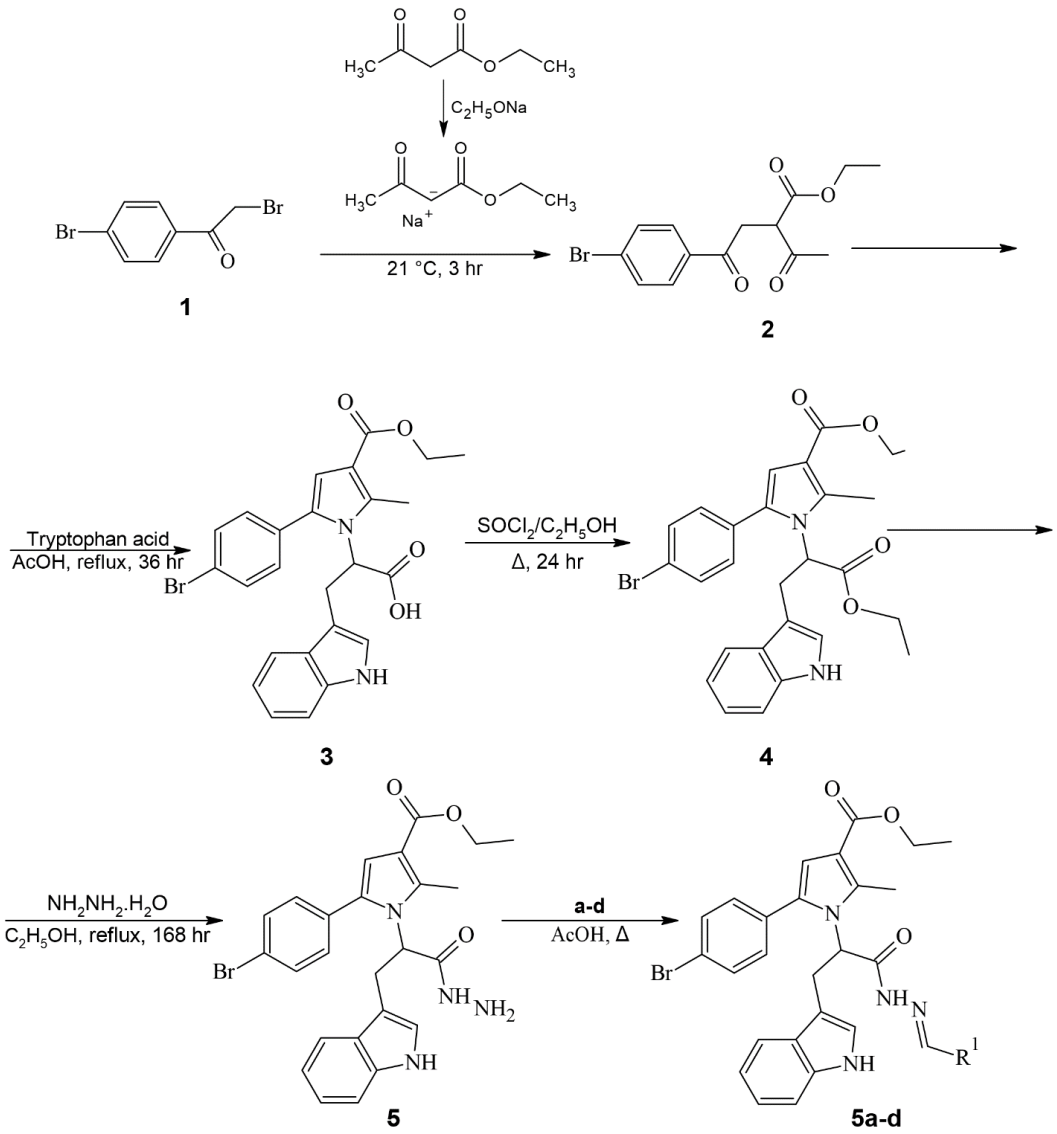
To calculate the physicochemical and pharmacokinetic properties of the most prominent antioxidants, the ADME test employing the QikProp module in Schrödinger (Schrödinger Release 2021-2: QikProp, Schrödinger, LLC, New York, NY, 2021) was performed. The latter virtual simulation examines ranges based on the properties of 95 % of the known drugs and evaluates outliers based on Lipinski's rule of five. [14]

3. RESULTS AND DISCUSSION

3.1. *Design and synthesis of pyrrole analogues*

Several studies have demonstrated the antioxidant potential of the hydrazide-hydrazones moiety.[15,16] Implementing electron donating groups has also been discussed as a potential technique for enhancing the overall antioxidant properties.[17] In addition, research papers have described good antioxidant capacities of the pyrrole moiety, with particular attention given to the multi-functionalized pyrrole rings.[18,19] Considering the aforementioned observations, we designed and synthesized one multi-substituted pyrrole molecule, its corresponding hydrazide, and four hydrazide-hydrazones containing electron donating groups. A tryptophan motif was also present in the title molecules considering its antioxidant capacity.[20]

The *N*-pyrrolylcarboxylic acids were synthesized following the classical Paal-Knorr approach with several modifications.[21] In the current work, the amino acid tryptophan was utilized for the condensation reaction with ethyl 2-acetyl-4-(4-bromophenyl)-4-oxobutanoate (**2**). The latter intermediate dicarbonyl compound (**2**) was produced by C-alkylation of the commercially available 2,4'-dibromoacetophenone (**1**) and ethyl 3-oxobutanoate. A hydrazinolysis with excess hydrazine-hydrate was also carried out to obtain the desired *N*-pyrrolylhydrazide (**5**). For the production of the pyrrole-based hydrazide-hydrazones (**5a-d**), we utilized equimolar concentrations of the hydrazide **5** (Scheme 1) and the corresponding carbonyl partners (**a-d**) (Figure 1).



Scheme 1. General scheme for the conventional synthesis of pyrrole-based hydrazide-hydrazones

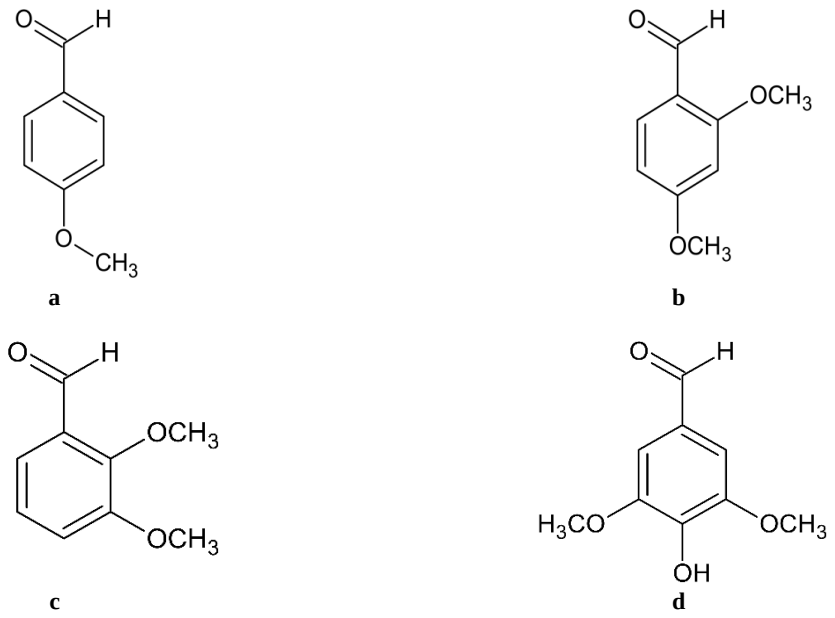


Fig. 1. Ketone-based fragments utilized for the condensation reaction

Since its introduction in the field of synthetic chemistry, the application of MW irradiation has been growing exponentially.[22] Essentially, the MW irradiation achieves high temperatures due to the direct heating effect.[23] Appling MW heating leads to numerous improvements compared to the conventional conditions. Therefore, in the current work, the conventional synthetic reactions of compounds **3, 4, 5,** and **5a-d** were optimized by applying microwave heating. After the utilization of MW-assisted synthesis, the reaction times and the yields were significantly increased (Table 1).

The microwave-assisted Paal-Knorr condensation of compound **2** with tryptophan led to a significantly reduced reaction time (55 min) and a higher yield of 89 % compared to the conventional heating. A drastic change in the synthetic time was observed during the hydrazinolysis of the ester (**4**). The latter reaction required 2 h of MW irradiation, while the conventional heating completed the reaction in 168 h (7 days). Importantly, all hydrazide-hydrazones were prepared for 30 s with excellent yields when the MW radiation was employed in the process.

T a b l e 1

Reaction times and yields after applying conventional and MW heating

Compound	Reaction time		Yield (%)	
	Microwave synthesis	Conventional synthesis	Microwave synthesis	Conventional synthesis
3	55 min	36 h	89 %	67 %
4	40 min	24 h	96 %	82 %
5	2 h	168 h	87 %	59 %
5a	30 s	30 min	92 %	72 %
5b	30 s	40 min	91 %	64 %
5c	30 s	50 min	94 %	75 %
5d	30 s	50 min	88 %	64 %

3.2. *Antioxidant activities*

Two methods were applied to assess the free-radical scavenging capacities of the title compounds – ABTS and DPPH assays.

3.3. *DPPH assay*

The scavenging activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is attributed to the hydrogen donating ability of antioxidants with a subsequent decrease in the absorbance at 517 nm. The DPPH radical scavenging activity of the title hydrazid-hydrazone derivatives was determined at three concentrations ranging from 31 to 250 μM. The antioxidant effects of compounds **3, 5,** and **5a-d** were examined, and the results were compared with those of Trolox used (Figure 2).

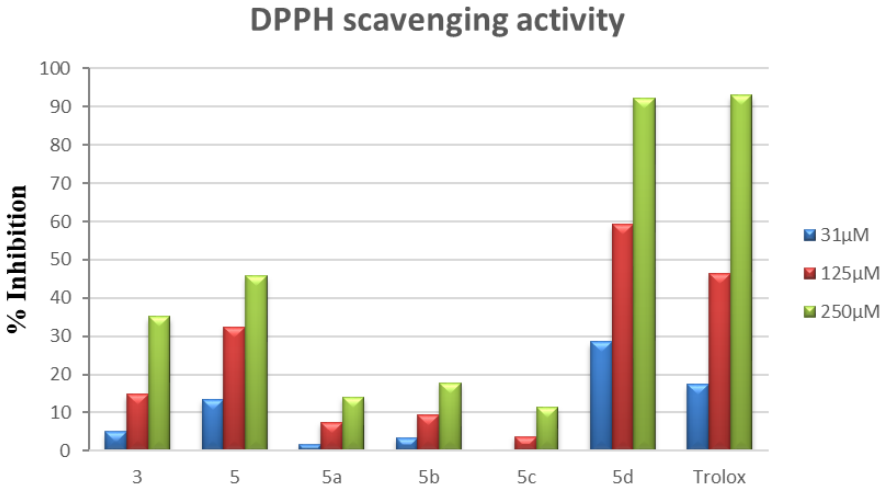


Fig. 2. DPPH radical capacities of the title compounds at concentrations of 31 to 250 μM. Standard deviation (SD) $n = 3$.

The highest DPPH scavenging activity was achieved by the hydrazide-hydrazone **5d**. The latter compound demonstrated better antioxidant capacity than Trolox. Inhibitory percentages of 92.2, 59.2, and 28.25 % were detected at concentrations of 250, 125, and 31 μM, respectively. Compounds **3** and **5** also demonstrated good DPPH scavenging activities; however, their inhibitory values were drastically lower. When only methoxy moieties were present in the benzaldehyde group (**5a, 5b,** and **5c**), the antioxidant effects were reduced.

3.4. *ATBS assay*

During the ABTS assay, the evaluated antioxidant neutralizes the ABTS cation, which could be detected by the discoloration of the initial color. Low absorbance at 734 nm indicates that the compound possesses antioxidant capacity. The ABTS antioxidant assay of the title compounds is given in Figure 3.

The results demonstrated that the hydrazide-hydrazone containing the 3,5-dimethoxy-4-hydroxybenzene moiety (**5d**) is the most promising antioxidant out of all the tested compounds. The latter molecule showed higher ABTS scavenging capacity than the standard – Trolox. At concentrations of 250 μM, 125 μM, and 31 μM, compound **5d** demonstrated inhibition of 94.8, 84.1, and 49.9 %, respectively. The former observation agreed with the results delivered from the DPPH experiments.

Moreover, moderate results were demonstrated by the hydrazide (**5**) and the hydrazide-hydrazone comprising 4-methoxybenzene moiety (**5a**). A recent study showed that 4-methoxy derivatives of hydrazide-hydrazones possess high antioxidant potential,[15] which was not in good agreement with this study.

Several advantages of ABTS over DPPH, such as the ability to examine both hydrophobic and hydrophilic compounds, as well as to test bulky structures, have been previously described.[24,25] The discussed results in this study showed better performance from the ABTS test which confirms the differences between the two tests.

Analyzing the data from both antioxidant bioassays, some common characteristics could be observed. The application of a hydroxyl moiety in the benzaldehyde utilized for the synthesis of the corresponding hydrazide-hydrazones is essential for the overall antioxidant properties of the compounds. When only methoxy groups were included in the final structure, the radical scavenging capacity was drastically reduced.

Interestingly, the *N*-pyrrolecarboxylic acid (**3**) and the *N*-pyrrole hydrazide (**5**) showed better radical-scavenging activity than most of the hydrazide-hydrazones (**4a**, **4b**, and **4c**). In addition, the implementation of a tryptophan moiety increased the overall antioxidant properties of the pyrrole-based compounds compared to recently synthesized analogs.[26] This is in good agreement with data showing the good antioxidant capacity of the tryptophan acid.[20]

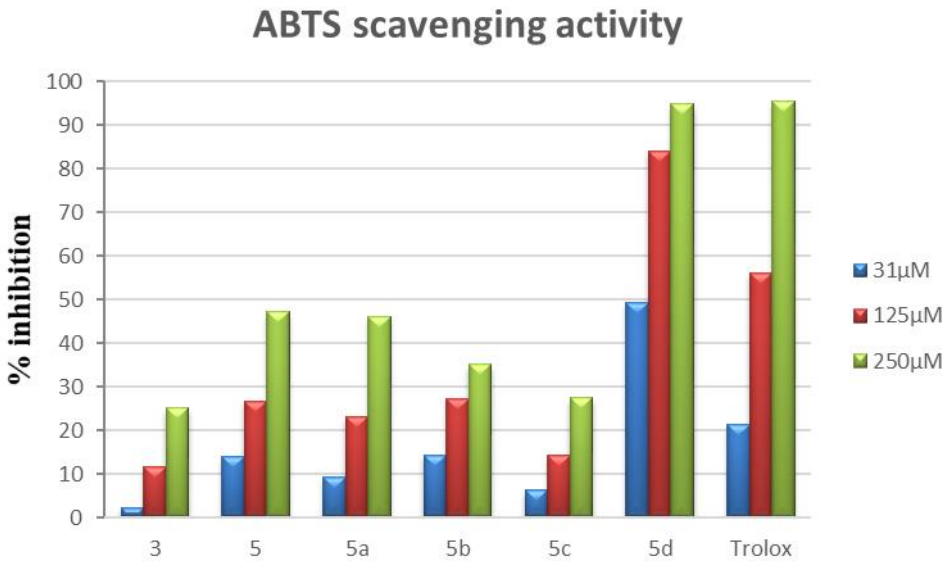
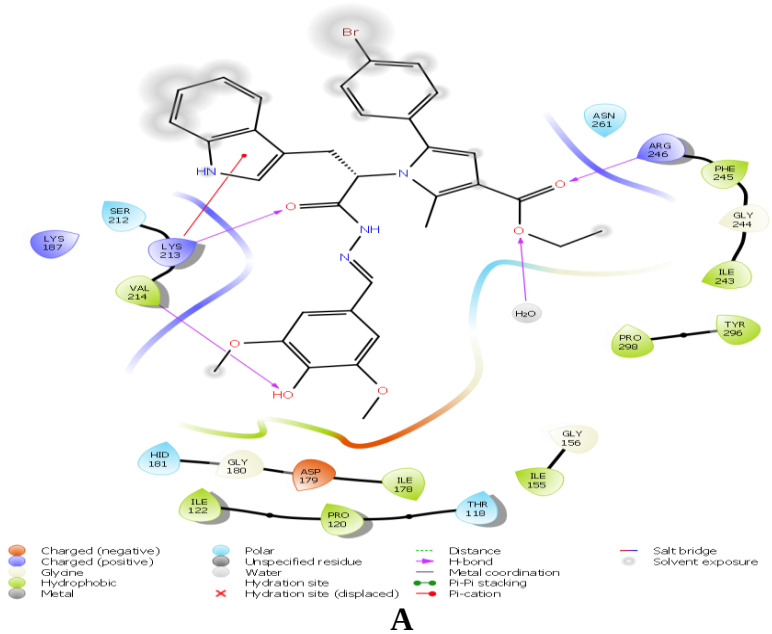


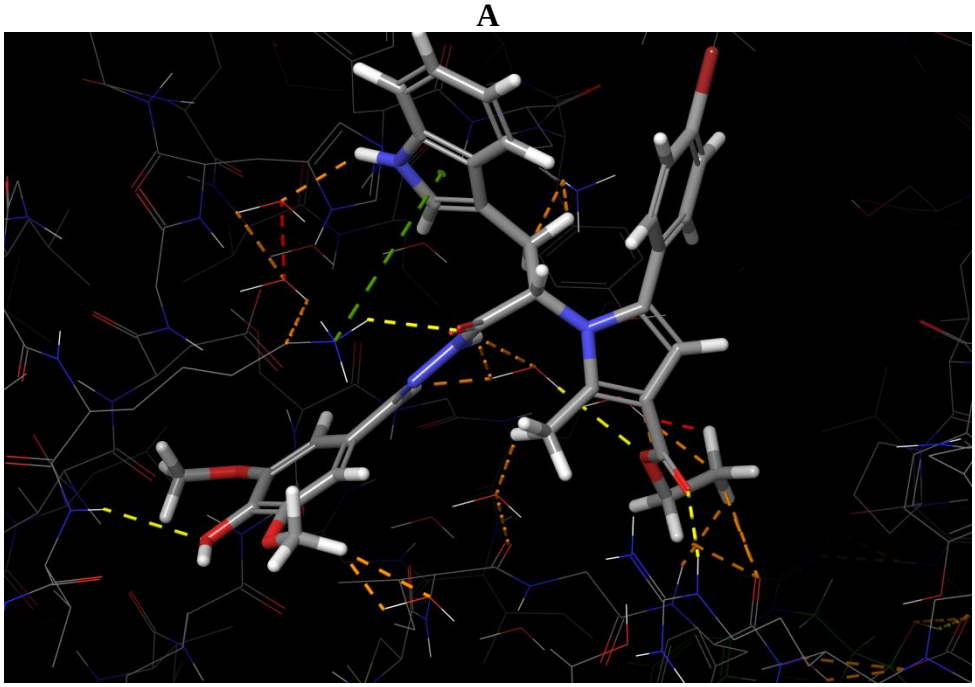
Fig. 3. ABTS assay of the title compounds at concentrations of 31 to 250 μM. Standard deviation (SD) $n = 3$

3.5. *Molecular docking studies*

NADPH oxidase (NO) is a family of enzymes whose main function is to transfer electrons to molecular oxygen. The latter process could then lead to the generation of ROS.[27] NO is known to generate ROS during the metabolism of arachidonic acid, and their inhibitions break the ROS production cycle with the consequent reduction of the oxidative stress and maintenance of redox homeostasis[28]. Several papers have reported molecular docking of compounds with free-radical scavenging activities in the active site of NO.[29,30] Initially, the co-crystallized ligand of **2CDU** (adenosine-5'-diphosphate) was re-docked back into the active site. The resolution was under 2 Å, which validated the docking protocol. Thereafter, the most active hydrazide-hydrazone **5d** was docked in the active site of **2CDU**. **5d** interacted with **2CDU** by forming various stable intermolecular bonds with its active amino acids. The interactions are provided in 2D and 3D forms in Figure 4.



A



B

Fig. 4. Major interactions between the most potent radical-scavenging compound **5d** and the active site of NO. A) 2D interaction; B) 3D interaction

Three strong hydrogen bonds were observed. The first one was formed by the active amino acid Arg246 and the carbonyl group of the ester fragment located at the third place in the pyrrole ring (length of 2.02 Å). The second hydrogen bond was formed between the carbonyl group of the hydrazide moiety and amino residue Lys213 (2.08 Å). Importantly, the third hydrogen bond was formed between Val214 and the hydroxyl group located in the benzene ring (2.54 Å). The latter interaction might be related to the significantly higher antioxidant activity of **5d** when compared to the hydrazide-hydrazones, which lack a hydroxyl group in their molecules. A water mediated hydrogen bond was established between one of the active waters and the ester fragment in the pyrrole ring. In addition, the active residues Pro120, Ile155, Lys213, and Ile243 formed hydrophobic interactions with the ligand **5d**.

3.6. *ADME prediction*

As a final stage of our study, an *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis was carried out. The pharmaceutically relevant properties of the most prominent title compounds were examined. The QikProp module in Maestro 11.8 was utilized for the determination of the ADMET of compounds **3, 5,** and **5d**. The results are compiled in Table 2.

T a b l e 2

ADME properties of the most promising multi-target inhibitors

Compound	[a] MW	[b] Acc HB	[c] Donor HB	[d] QPLog Po/w	[e] QPLog S	[f] QPLog BB	[g] % Human Oral Absorption	[h] PSA	[i] Rule of Five	[j] Metab
3	495.37	2	4	6.26	–7.76	–1.08	88 %	93.97	1	3
5	509.40	4	5	4.51	–6.15	–1.40	82 %	112.55	1	3
5d	673.56	3	6.75	7.62	–10.23	–1.87	92 %	130.25	2	6

[a]Molecular weight of the molecule (range:130.0 – 725.0); [b]Number of hydrogen bond acceptors (range: 2.0 – 20.0);
[c]Number of hydrogen bond donors (range: 0.0 – 6.0); [d]Predicted octanol/water partition coefficient (range: − 2.0–6.5);
[e]QplogS: Predicted aqueous solubility (range -6.5 – -0.5); [f]QPlogBB: Predicted brain/blood partition coefficient (range: – 3.0 – 1.2);
[g]Percent Human Oral Absorption; [h]PSA: Van der Waals surface area of polar nitrogen and oxygen atoms (range: 7.0 – 200.0);
[j]Number of violations of Lipinski's rule of five (Range: maximum is 4); [j]Number of likely metabolic reactions (Metab)

As per QikProp ADME analysis, none of the compounds obey the rule of five. The most potent antioxidant, **5d**, violates two of the principles of Lipinski's rule of five. Moreover, the blood-brain barrier partition coefficient is moderate, and further improvements in that area could be conducted. Compounds **3** and **5** demonstrated lower oral absorption rates of 88 % and 82 %, respectively. However, both compounds are expected to have low numbers of metabolites, and they violated only 1 of Lipinski's principles.

4. CONCLUSION

Six novel *N*-pyrrolyl compounds were synthesized by MW and conventional heating. Their radical scavenging capacities were observed by DPPH and ABTS assays. Molecular docking simulations and in silico ADME provided additional data about the most active molecules.

After comparing the conventional and the microwave heating, we observed that the latter is drastically outperforming the classical approach in the synthesis of hydrazide-hydrazones. The free-radical scavenging assays demonstrated that the hydrazide-hydrazone pyrrole-based compound comprising the 4-hydroxy moiety (**5d**) is the most prominent. Moreover, the latter ligand formed a stable complex with the active site of NO. The virtual simulation demonstrated that the hydroxyl moiety plays an important role in the antioxidant activity of the hydrazide-hydrazone. Moreover, the ADME data revealed that the title compounds possess good physicochemical properties. Further investigations in that area could be conducted to observe the effects of electron-withdrawing groups on the antioxidant effect of the title hydrazide-hydrazones.

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