REVIEWER 2

Chalcone and flavanone derivatives are common known from their broad spectrum of biological activity, what is well documented in literature. Particularly interesting are results of their activity as potential inhibitors of acetylcholinesterase. This publication seems to be within the scope of journal. However it needs several corrections to be more acceptable for publication.

1) In introduction and in conclusion, short explanation of research novelty is needed.

R: Attending this petition, both Introduction and Conclusion were modified to include the requested information.

| 1. Introduction | Pharmacological effects of flavonoids are related to their antioxidant activity, as they are powerful antioxidants against free radicals and ROS [12]. Structurally, the free radical scavenging capacity is primarily attributed to high reactivity of their hydroxyl substituents that participate in the reaction [25]. In this context, we report the chemical synthesis of chalcone and flavone analogs with different substituents on the respective B ring (Figure 1). In addition to the mandatory molecule characterization, their antioxidant and AChE inhibition activity was evaluated by in vitro testing, to get preliminary insights regarding the structure-activity relationship, allowing us to further develop the continuous search for new therapeutic agents. |
| 4. Conclusions | On the other hand, in the AChE inhibition assay, the better results from the chalcone and flavone families (compounds 2d and 3d) share the nitro functional group, with IC\textsubscript{50} values of 21.5 and 26.8 µg/mL respectively. Docking results suggest the principal interactions compounds 2d and 3d have with AChE active site are mostly π-π stackings. This can be observed with residues Trp84 and Tyr334 from the CAS and PAS, known binding zones of the enzyme. Although further in vivo testing must be performed, our results represent an important step towards the identification of improved antioxidants and acetylcholinesterase inhibitors. |
2) In figure 1 carbon atoms should be numbered and also in structure of first compound in supplementary information.

R: In response of the accurate petition of the reviewer, Figure 1 in the manuscript, as well as in the supplementary material, has been modified, so in both cases the molecule skeletons are numbered.

![Figure 1](image)

**Figure 1.** Basic structure and B ring position of chalcone (a) and flavone (b) molecules.

3) In Materials and Methods detailed description of GC-MS method is necessary (carrier gas, detailed information about capillary column, kind of detector, temperature of detector and temperature of injector, temperature programme, etc.)

R: Regarding this observation, section 2.1 of manuscript has been modified to include the description of GC-MS method, as can be seen as follows:

### 2.1. General Information

All commercial reagents and solvents were used as received and did not required any purification before use. Melting points were taken on a Mel-Temp melting point apparatus (Thermo Scientific). UV spectra were recorded on UV-VIS spectrophotometer model type Genesys 20 and expressed in nm. FT-IR spectroscopic studies were carried out on a FT-IR spectrophotometer Nicolet is5 (Thermo Scientific). NMR spectra were recorded on a Bruker Avance III spectrometer 400 MHz. The chemical shifts (δ) are presented with tetramethylsilane (TMS) (δ: 0.00) as the internal standard. Gas chromatography-Mass spectrometry data were recorded on a Thermo Scientific TRACE 1310 (GC) and Thermo Scientific single quadrupole ISQ LT (MS), with a column model TG-SQC (30 m × 0.25 mm inner diameter, 0.25μm film thickness). The detector temperature was 240°C, injector temperature was 250°C and transfer-line temperature was 250°C; oven temperature started at 120°C for 1 min, increased at a 40°C/min rate until 280°C, with a hold time of 10 min. Helium was employed as carrier gas, at 1 mL/min flow with split ratio 1:20. Column chromatography purifications were carried out on Silica Gel 60Å (Sigma-Aldrich, 230–400 mesh). The purity of compounds was checked by thin-layer chromatography (TLC) carried out on aluminum backed silica plates by Merck and plates were revealed using a UV 254 nm light.
4) In Materials and Methods detailed information about used eluent in column chromatography should be given.

R: Information concerning the compounds purification in Materials and Methods section has been extended, attending the reviewer observation.

<table>
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<tr>
<td><strong>2.2. Synthesis of Chalcone Derivatives</strong></td>
<td>The precipitate was filtered, washed with cold water, dried and purified by column chromatography. All structures were confirmed by mass and NMR spectra as discussed below.</td>
<td>The precipitate was filtered, washed with cold water, dried and purified by column chromatography, employing eluent mixtures of n-hexane/ethyl acetate and dichloromethane/ethyl acetate in different proportions; some products were purified by recrystallization with a methanol/H₂O (1:2) mixture. All structures were confirmed by mass and NMR spectra as discussed below.</td>
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| 2.3. Synthesis of Flavone Derivatives | The products (3a-3k) were then extracted with ethyl acetate and purified by column chromatography. | The products (3a-3k) were then extracted with ethyl acetate and purified by column chromatography, employing eluent mixtures of n-hexane/ethyl acetate, dichloromethane/ethyl acetate and ethyl acetate/methanol in different proportions; some products were purified by recrystallization with a methanol/H₂O (1:2) mixture. |

5) The numbering of carbon atoms in Figure 1 should be used to assign NMR signals to concrete protons.

R: Based on the numbering added to Figure 1, all signals of NMR (¹H and ¹³C) were assigned on sections 2.2 and 2.3 of the manuscript.

6) Line 367 “trans” should be in italic.

R: Accordingly with this observation, the text was modified, resulting as it follows:

The vinylic protons of the α,β-unsaturated system are present as doublets for Hα=7.48-8.10 ppm and Hβ=7.69-8.89 ppm, being the coupling constants JHα-JHβ = 14.9-15.8 Hz, which indicates a **trans** configuration for these protons. All the aromatic protons were observed at
their expected shifts, so as their coupling constants.

7) **Line 416** What is the reason, that in this research methoxyl groups are not an important factor for the antioxidant activity of flavone derivatives?

**R:** In response to this observation, we extended the section 3.2 to be clearer in the effect of methoxyl groups in the antioxidant activity:

The influence of electron-donor groups like methoxyl can be analyzed with the 2e (p-OMe), 2f (m-OMe) and 2g (p,m-OMe) analogs; the presence of these groups did not favor the activity, getting concentrations of 100, 170 and 5x10^4 μg/mL respectively in DPPH assay, having a similar behavior of the flavones 3e-g, with EC_{50} of 30, 39 and 410 μg/mL. These results may appear to contrast with the reported by many authors where methoxyl groups are considered an important factor for the antioxidant activity [41-42], however, this is the case where methoxyl groups help with the lipophilicity of the molecule, for example, when the antioxidant activity is measured by the lipid peroxidation activity assay [39]. When employing DPPH test, it is required the transfer of acid protons; this implies that just as in the case of the chalcones, we consider indispensable the presence of at least one hydroxyl group, explaining the lack of scavenging activity for the synthesized compounds with only methoxyl groups (3e-g).

8) **Line 448, line 452, line 459:** Year of publication should be removed.

**R:** In response to this suggestion of the reviewer, changes were made in text, resulting as it follows:

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<td>448</td>
<td>Y. Sugiyama et al. (1996) reported the importance of diketonic systems in the antioxidant activity, both in their keto or enol form [52].</td>
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<td>452</td>
<td>The acetylcholinesterase inhibitory activity of the synthesized compounds was evaluated employing Adewusi adaptation of Ellmans spectrophotometrical assay (2011) [30], using galantamine as a reference.</td>
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<td>459</td>
<td>Hasan et al. (2005) [54] reported that hydroxyl groups in ortho position of the A ring of chalcones, are an important structural element in the AChE inhibitory activity;</td>
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9) In text of manuscript should be emphasized that galantamine is a drug used in treatment of Alzheimer disease.

R: In response to the suggestion made by the reviewer, section 2.5 was modified as can be seen as follows:

Six wells of each plate were employed without a compound to test, to serve as a 100% enzymatic activity control. The increase of absorbance due to spontaneous substrate hydrolysis was corrected subtracting the absorbance before the enzyme addition from the absorbance obtained after the enzyme addition. Inhibition percentage was calculated by the following equation:

\[
\text{inhibition } \% = 1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Where \(A_{\text{sample}}\) is the absorbance difference between time 0 and 225 s in presence of any compound to test or inhibitor, and \(A_{\text{control}}\) is the absorbance difference between time 0 and 225 s of the 100% enzymatic activity control. Inhibitory concentration 50 (IC\(_{50}\)) was calculated by interpolation from the graphic of inhibition percentages in function of the employed concentrations. All experiments were performed by triplicate and galantamine was employed as a positive control, which is a drug currently used for Alzheimer disease treatment. [12]

10) Obtained results of AChE inhibition should be discussed with results described in publication of Sukumaran et al. Molecules, 2016, 21, 955.

R: Attending this petition from the reviewer, we added a paragraph with this analysis to the section 3.3:

While we can notice a general improvement of the results with the presence of hydroxyl and methoxyl groups in the chalcones B ring, Sukumaran et al. [56] mentions that in 2'-hydroxychalcones the AChE inhibition is generally favored with halogens in the B ring. Nevertheless, their chalcone with chlorine in \(p\)-position of the B ring did not showed significant activity, molecule that corresponds with our compound \(2h\), from which we neither detect activity in the tested concentration scale.

11) References should be formatted according to the requirements of the journal, for example, DOI number instead of CrossRef.

R: Adjustments were made to the References section to comply this request, which can be observed in the manuscript.