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Proceeding Paper

Use of Oxidative Coupling Strategy as a Means to Increase In Vitro Antioxidant Activity of Vanillin Derivatives †

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Abstract: The aim of this study was to investigate the antioxidant properties in vitro of three different vanillic dimmers (Compounds **1a-c**). They were synthesized through an oxidative coupling strategy in good yields. The targeted compounds were found to be highly active for the total antioxidant assay, as well as for the lipid peroxidation test. All investigated compounds exhibited superior or comparable antioxidant capacity in comparison to precursor vanillin, proving that the oxidative coupling is a great strategy to increase antioxidant behavior of vanillin derivatives.

Keywords: vanillic dimers; oxidative coupling; antioxidant activity

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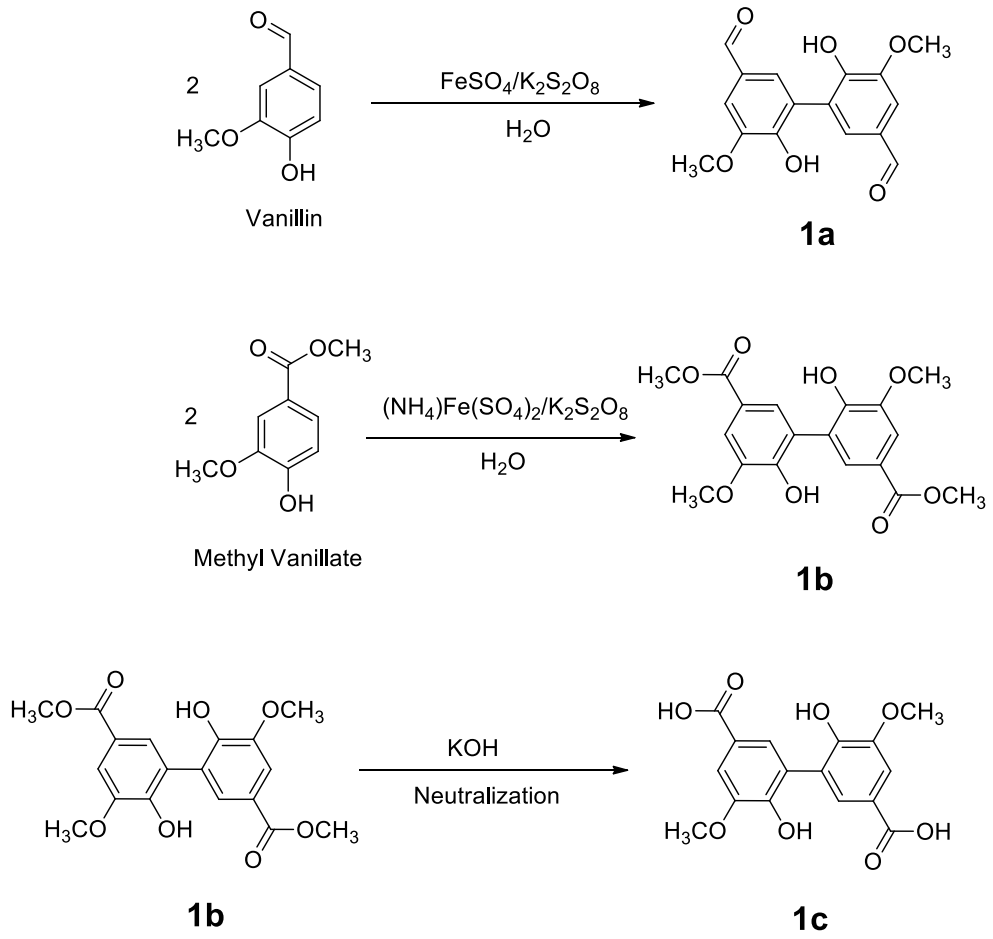
1. Introduction

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the organism ability to face their action by antioxidant protection [1,2]. This imbalance has been linked to numerous chronic affections, especially cardiovascular and oncological diseases [3,4]. Adding low concentrations of antioxidants can effectively quench the reactive oxygen species before they attack biomolecules causing damage [5]. Vanillin is one of the most popular natural flavoring agents and is widely used in food and cosmetic industry, and their antioxidant properties have been extensively studied [6]. Nowadays synthetic antioxidants are increasingly in demand [7]. Thus, it is important to create synthetic compounds derived from natural molecules that they could increase the antioxidant activity of their natural analogues. Hence, in this work we use the oxidative coupling as a strategy to improve, through functionalization, the antioxidant behavior of vanillin. For this series of compounds, we evaluated their antioxidant activity *in vitro*-through three different assays.

2. Materials and Methods

2.1. Synthesis of Vanillic Dimers

Oxidative coupled vanillic dimers **1a-c** were prepared as depicted in Scheme 1 according to methods found in the literature [8–10].



Scheme 1. Preparation of compounds **1a-c**.

To obtain compound **1a**, 0.89 mmol of $FeSO_4$ and 12.51 mmol $K_2S_2O_8$ were added under stirring to an aqueous solution of vanillin (23.33 mmol). The mixture was heated at 50 °C for 120 h until the reaction was complete as indicated by TLC assay. After cooling at room temperature, the crude product was filtered and washed with cold ethanol and dried in vacuum oven at 40 °C during 24 h.

For **1b** preparation, 6 mmol of methyl vanillate were dissolved in 200 mL distilled water and the mixture was heated until total dissolution. Once the esther was dissolved, 0.3 mmol of $(NH_4)_2Fe(SO_4)_2$ and 3.0 mmol of $K_2S_2O_8$ were added, the heating was kept for 3 min and then heating was turned off, and kept stirring for another 30 min. The product was filtered and washed with 300 mL hot water and 300 mL cold water, and dried in vacuum oven at 40 °C during 24 h.

For the synthesis of compound **1c**, 1.37 mmol of **1b** was suspended in a THF:water mixture (15 mL, 1:1 v:v) and 13.7 mmol of KOH pellets were added. The resulting two phases were heated to 70 °C and stirred vigorously during 16 h.

After cooling to room temperature, organic phase was removed and aqueous phase was acidified with a HCl solution (6 M) until pH = 1 was reached. The solid product was collected, washed with water until neutrality and dried in vacuum oven at 40 °C during 24 h.

2.2. Evaluation of Antioxidant Properties

Compounds **1a-c** synthesized were studied for their antioxidant properties using three assays with differentoxidative potential evaluation mechanisms.

Stock solutions (10 mg/mL) of targeted compounds **1a-c** were prepared in DMSO. For all the experiments, different dilutions of these solutions in ethanol were performed.

2.2.1. Reducing Power Method (RP)

In this method, described by Oyaizu [11], a colored complex is formed with $K_3Fe(CN)_6$, CCl_3COOH and $FeCl_3$. Increase in the absorbance of reaction mixture indicates the reducing power of samples. 1 mL of targeted compound (20–50 μg/mL) was placed in a tube was mixed with 3 mL of buffer phosphate (pH 6.6) and 3 mL of aqueous solution of $K_3Fe(CN)_6$ (1% *w*/*v*). The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 mLCCl_3COOH (10% *w*/*v*). Finally, 2.5 mL of this mixture was collected and mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1% *w*/*v*). The absorbance was measured at 700 nm against blank sample through time.

2.2.2. Nitric Oxide Scavenging Activity

Sodium nitroprusside is known to decompose in aqueous solution at pH 7.2 producing NO radicals. The method described by Marcucci et al. [13] was used, and it is based in the decomposition of sodium nitroprusside at physiological pH producing nitric oxide radicals. Under aerobic conditions, NO radical reacts with oxygen to produce stable products, the quantities of which can be determined using Griess reagent. For this, 2 mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer (pH 7.4) is mixed with 0.5 mL of sample at various concentrations (0.4–1.0 mg/mL). The mixture is then incubated at 25 °C. After 150 min of incubation, 0.5 mL of incubated solution is withdrawn and mixed with 0.5 mL of Griess reagent. (0.5 g of sulphanilamide, 1.25 mL orto-phosphoric acid and 0.05 g N-1-(Naphthyl)ethyl-enediamine). The mixture is then incubated at room temperature for 30 min and its absorbance is measured at 546 nm. The amount of nitric oxide radical inhibition is calculated following Equation (1).

$$\text{Inhibition of NO radical (\%)} = [Ac - As)/Ac \times 100 \quad (1)$$

where A_c is the absorbance of the control, and A_s is the absorbance of the sample.

2.2.3. Thiobarbituric Acid Reactive Substances Method (TBARS)

The TBARS assay is used to evaluate lipid peroxidation. In this test,malondialdehyde (MDA) is measured, which results from oxidation of lipid substrates. At low pH and high temperature (100 °C), MDA binds with thiobarbituric acid to form a red complex that can be measured at 532 nm. The increased amount of the red pigment formed correlates with the oxidative rancidity of the lipid.

The method of Ottolenghi [12] with modifications was used. A volume of 4 mL of targeted compound (1 mg/mL) was placed in a tube along with 4.1 mg of 2.52% oleic acid in absolute ethanol, 8 mL of 0.05 M phosphate buffer (pH 7.0) and 3.9 mL water. The mixture was placed in an oven at 40 °C in the dark. To 1 mL of this solution, 2 mL of trichloroacetic acid (20%) and 2 mL of thiobarbituric acid solution (0.67%*w*/*v*) were added and then incubated. The mixture was placed in a boiling water bath for 10 min, and the absorbance was measured at 532 nm.Antioxidant activity was determined using Equation (2).

$$\text{Inhibition (\%)} = [(A_c - A_s)/A_c] \times 100\% \quad (2)$$

where A_c is the absorbance of the control, and A_s is the maximum absorbance of the sample on the same day.

3. Results

3.1. Preparation of Vanillic Dimers

Preparation of targeted vanillic dimmers **1a-c** using oxidative coupling as strategy was easily achieved. The three synthesized compounds were solid and their yields, m.p. and NMR data are in accordance with reference methods [8–10].

3.2. Antioxidant Properties

The antioxidant properties of the obtained compounds **1a-c** were tested. The results of reducing power assay are shown in Figures 1 and 2. These results show that vanillic dimmers **1a-c** have reduction potentials higher than their natural analogue vanillin.

Also, it can be observed that the antioxidant activity measured by this method was correlated to the concentration of vanillic dimers. The highest absorbance values were observed when compound **1c** was used.

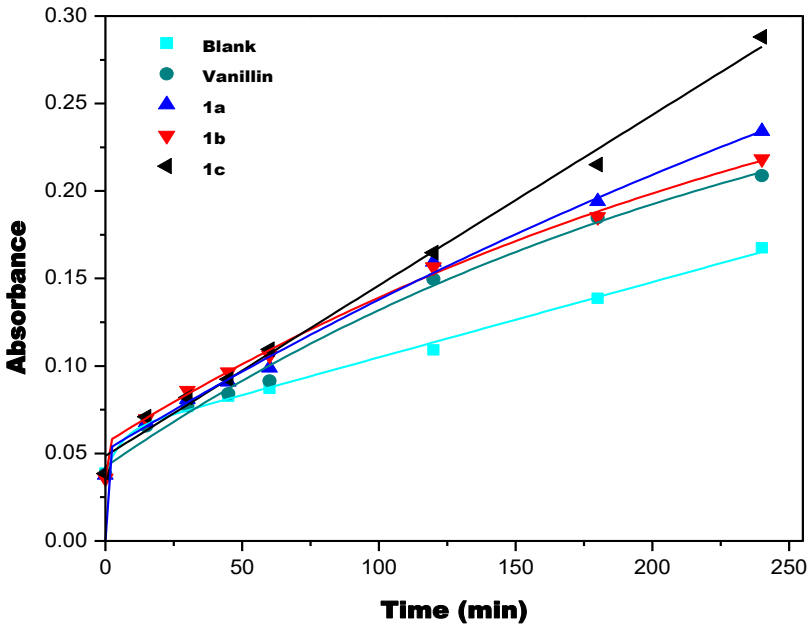


Figure 1. Reducing power of compounds **1a-c** (20 μg/mL) versus time.

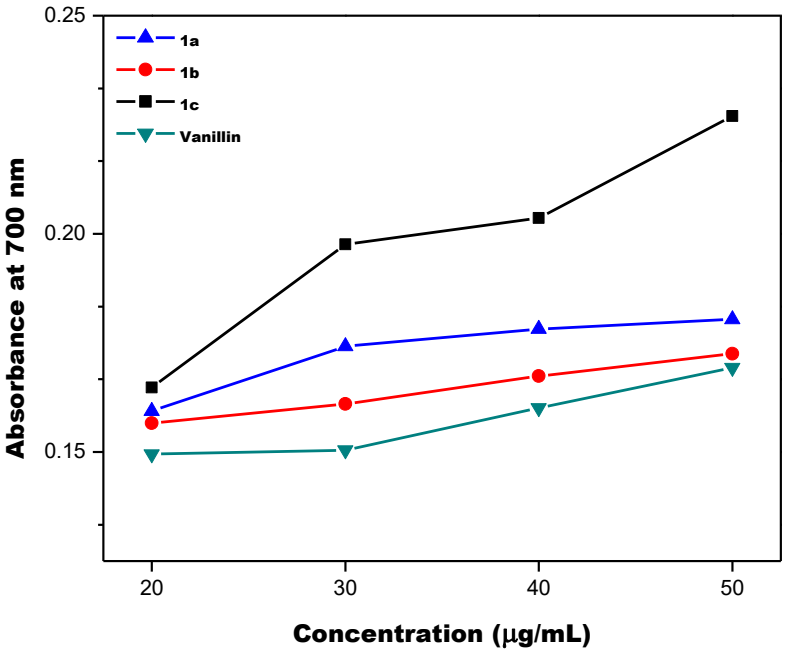


Figure 2. Reducing power of compounds **1a-c** at different concentrations (20–50 μg/mL) (t = 120 min).

Subsequently, the nitric oxide scavenging activity was evaluated. As it can be observed in Figure 3, all compounds including vanillin presented similar inhibitory percentages indicating the effective scavenging nitric oxide activity of these compounds.

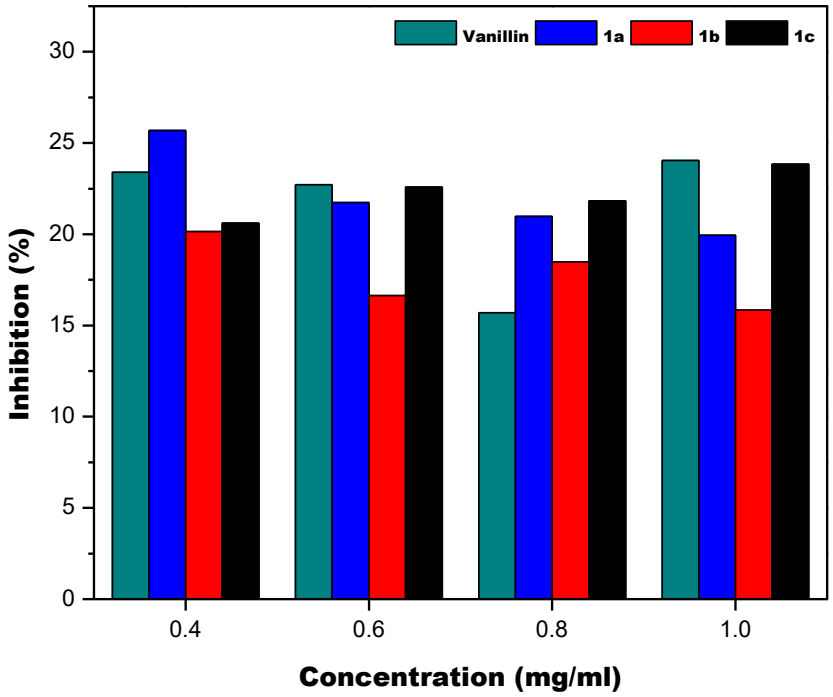


Figure 3. Nitric oxide scavenging activity of compounds **1a-c** and vanillin at different concentrations (0.4–1.0 mg/mL).

Also, the antioxidant properties of compounds **1a-c** were evaluated using the thiobarbituric acid reactive substance method. The oxidation of oleic acid was investigated showing that in presence of compounds **1a** and **1c**, the inhibition percentages of oxidation products are higher than their natural analogue vanillin. This proved that these compounds present inhibitory activity towards lipid peroxidation.

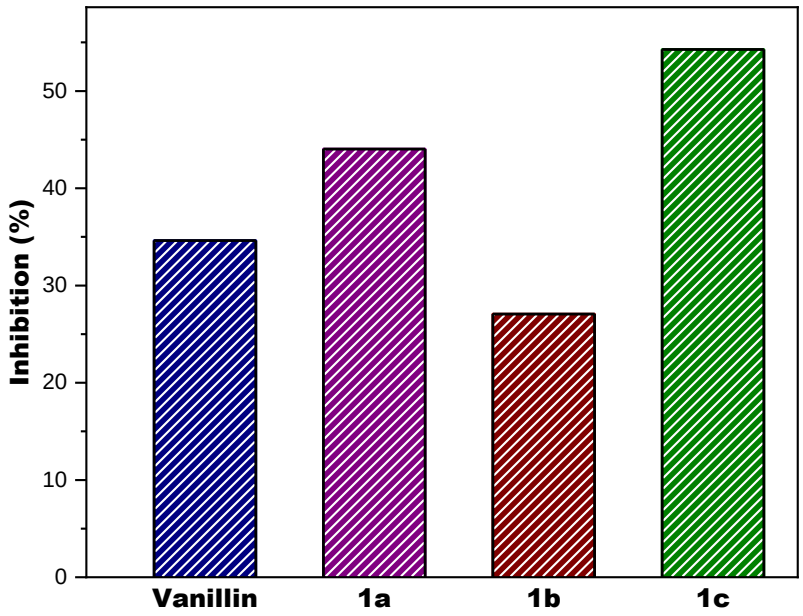


Figure 4. Antioxidant properties of compounds **1a-c** evaluated using thiobarbituric acid reactive substance method.

4. Conclusions

In this study, the synthesis and the antioxidant activity of three diferentvanillic dimers is described. All investigated compounds exhibited superior antioxidant capacity in comparison to precursor vanillin, proving that the oxidative coupling is a great strategy to increase antioxidant activity of vanillin derivatives. Among the three dimers studied, compound **1c** possesses the best antioxidant properties among the studied dimers.

Author Contributions: Conceptualization, C.A.F. and L.G.G.; methodology, L.G.G.; formal analysis, A.P.R., L.G.G. and C.M.O.; investigation, C.A.F.; resources, C.A.F.; data curation, A.P.R., V.A.G.; writing—original draft preparation, L.G.G.; writing—review and editing, C.A.F.; supervision, V.A.G. All authors have read and agreed to the published version of the manuscript.

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