

Electrochemical Preparation and Evaluation of Cytotoxic Activity of Methoxyl-oxo-biseugenol, a New Oxidized Derivative of Biseugenol

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Electrochemical procedures have emerged as a powerful tool for the synthesis of complex organic molecules including transformation of natural products. In this study, the electrosynthesis of biseugenol in MeOH afforded one new oxidized derivative, which was characterized as methoxyl-oxo-biseugenol (MOB) by nuclear magnetic resonance (NMR) and electrospray ionization-high resolution mass spectrometry (ESI-HRMS) analysis. Since biseugenol was previously described as a cytotoxic natural product, MOB was also tested against tumoral cells B16F10-Nex2 (murine metastatic melanoma) and SKMEL-25 (human metastatic melanoma) as well as against non-tumoral cells MØ Raw 264.7 (murine macrophage immortalized) and HUVEC (human umbilical endothelium). As results, MOB showed inhibitory concentration that affects 50% of cells (IC₅₀) values of 9.5 ± 1.6 mg mL⁻¹ (B16F10Nex2), 13.2 ± 2.5 mg mL⁻¹ (SKMEL-25), 19.9 ± 3.5 mg mL⁻¹ (MØ Raw 264.7) and 36.3 ± 7.4 mg mL⁻¹ (HUVEC). Therefore, selectivity index (SI) values of MOB to tumoral cells B16F10Nex2 and SKMEL-25 were calculated as 2.1 and 2.8, respectively, higher than biseugenol (1.4 and 1.7, respectively). Based on these results, the superior cytotoxic activity of MOB in comparison to biseugenol could be associated, at least in part, to the presence of a non-aromatic ring and a conjugated carbonyl system instead of a phenol moiety.

Keywords: biseugenol, natural products, electrosynthesis, cytotoxic activity

Introduction

Eugenol, the major component in cloves oil, has been widely used as antimicrobial, antiseptic and antispasmodic agent.¹ Due to these properties, eugenol acts as an ingredient in cosmetics and pharmaceutical products.² Although there is a wide range of applications reported for eugenol,³ studies describing the action of its dimer, biseugenol, are scarce.⁴ Considering that the biphenyldiol unit, found in biseugenol, is also detected in a large number of natural compounds such as magnolol and honokiol which possess pharmacological properties,⁴⁻¹² it is expected that biseugenol also may exhibit potential biological activity. In this sense, a recent study¹³ has shown that biseugenol exhibits antiparasitic activity especially against *Leishmania amazonensis*

and *Trypanosoma cruzi*. Additionally, this dimer also showed antiproliferative activity in melanoma, which is one of the most serious forms of skin cancer, due to its ability to spread to other organs more rapidly if it is not treated at an early stage.¹⁴ Considering these aspects, investigations concerning biseugenol and its derivatives are of extreme importance and it is a field of study that needs to be extended and explored. In this aspect, oxidation is a powerful tool to synthesize and modify molecules, including natural products,¹⁵ which can be performed using different procedures. The first approach uses chemical oxidation which generally requires the use of toxic and mutagenic oxidizing agents, for example, chromate/dichromate or permanganate. A second and safer approach involves the use of electrochemical oxidation, with the products formed by the control of potentials and selective oxidation of part of the reagent.¹⁶ Due to these aspects, electrosynthesis follows the green chemistry principles.

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In memory of Dr Luiz Travassos due to his contribution to development of cancer research in Brazil.

Using this principle, electrochemical oxidation has been used to obtain several derivatives of natural products. Doi *et al.*¹⁷ have accomplished the synthesis of heliannuol E, which is typically isolated from *Helianthus annuus*. The synthesis was carried out by employing a novel ring-expansion reaction of spiro derivatives which were produced by electrochemical oxidation of the corresponding phenol. Ogami *et al.*¹⁸ performed the synthesis of gymnastatin A, which was isolated from the strain of *Gymnascella dankaliensis* separated from the sponge *Halichondria japonica*, through the efficient construction of spiro derivatives by electrochemical oxidation of the corresponding phenols. Cottet *et al.*¹⁹ performed the synthesis of two regioisomeric polycyclic xanthenes, 1,16- and 3,16-oxyguttiferone A, which were isolated from the seeds of *Symphonia globulifera*, together with its precursor, guttiferone A. The electrochemical oxidation of guttiferone A proved to be an efficient biomimetic method to generate xanthenes that exhibited cytotoxicity against the colon carcinoma (HCT 116) cell line. Studies^{20,21} involving electrochemical oxidation of eugenol have already been carried out, but none has previously been reported for biseugenol. It is worth mentioning that the electrochemical oxidation of eugenol leads to the formation of quinone units, which contribute to improving its antibacterial, anti-inflammatory, antifungal, antiviral, and antitumor activities.²²⁻²⁶

In view of these aspects, in the present work was performed, for the first time, the exhaustive electrolysis of biseugenol. The reaction product was characterized as methoxyl-oxo-biseugenol (MOB) by analysis of nuclear magnetic resonance (NMR) and electrospray ionization-high resolution mass spectrometry (ESI-HRMS) spectra. Finally, biseugenol and its oxidized derivative (MOB) were evaluated against two melanoma tumor cells (B16F10-Nex2 (murine metastatic melanoma) and SKMEL-25 (human metastatic melanoma)) as well as against non-tumoral cells (MØ Raw 264.7 (murine macrophage immortalized) and HUVEC (human umbilical endothelium)), in order to calculate the respective selectivity indexes (SI).

Experimental

General experimental procedures

Silica gel plates PF₂₅₄ (Merck, New Jersey, NJ, USA) was used for thin layer chromatography (TLC), while Sephadex LH-20 (Sigma-Aldrich, St. Louis, MI, USA) was used for column chromatography (CC). Analytical grade solvents (Labsynth, Diadema, SP, Brazil) were used for all extraction and chromatography procedures. Eugenol, at 99%

of purity, was purchased from Sigma-Aldrich (St. Louis, MI, USA). NMR spectra were obtained in a Bruker (Billerica, MA, USA) Ultrashield 300 Avance III spectrometer, operating at 300 MHz to ¹H and at 75 MHz to ¹³C nuclei, respectively. CDCl₃ and tetramethylsilane (TMS), both from Sigma-Aldrich (St. Louis, MI, USA), were used as solvent and internal standard, respectively. ESI-HRMS spectrum was measured in a Bruker Daltonics MicroTOF QII spectrometer, in positive mode. For electrochemical reactions, a potentiostat Model PGSTAT-30/Autolab (Utrecht, Netherlands) was used. High performance liquid chromatography (HPLC) was performed using a Dionex Ultimate 3000 (Sunnyvale, CA, USA) chromatograph with a UVD-DAD-170 V as the detector, using a Luna Phenomenex (Torrance, CA, USA) C₁₈ column (particle and pore size of 5 μm and 120 Å), 4.6 × 250 mm (analytical mode).

Preparation of biseugenol

Biseugenol was prepared following a procedure already described in the literature.^{2,27} First, a saturated solution of K₃[Fe(CN)₆] (32.9 g, 100 mmol) in 145 mL of H₂O was prepared. Afterwards, eugenol (16.4 g, 100 mmol) was dissolved in a mixture of acetone (160 mL), H₂O (80 mL) and NH₃.H₂O solution (250 mL, 27%). K₃[Fe(CN)₆] solution was transferred slowly (for one hour) into the flask containing the eugenol solution. The reaction medium was kept under stirring for 5 h at room temperature. After this stage, 250 mL of NH₃.H₂O solution (27%) were added and the reaction was kept under constant magnetic stirring at room temperature for more 8 h. Then, the reaction medium was acidified to pH 3 using HCl 36%. The obtained solid was filtered under vacuum and thoroughly washed with distilled H₂O. Crude product was recrystallized with a mixture of EtOH:H₂O 1:1 to afford 3.95 g (12 mmol) of a white solid (24% yield).

Cyclic voltammetry experiments

Cyclic voltammetry experiments were performed in a potentiostat using a one-compartment electrochemical cell. Biseugenol was dissolved in the electrolyte solution (0.1 mol L⁻¹ LiClO₄ in MeOH). A platinum electrode (Metrohm, São Paulo, SP, Brazil, geometric area = 2.0 mm²) was used as working electrode, a Pt wire as a counter electrode and an Ag/AgCl (KCl 3 mol L⁻¹) as a reference electrode. The voltammograms were registered at scan rate of 100 mV s⁻¹.

Exhaustive electrolysis of biseugenol

Biseugenol was electrolyzed at room temperature in a divided cell. The working electrode was separated from the

auxiliary electrode with the help of a porous disk. A glassy carbon electrode (geometric area = 1 cm² each side) was used as a cathode, Pt spiral and Ag/AgCl (KCl 3 mol L⁻¹) were used as counter and reference electrodes, respectively. Biseugenol (100 mg) was solubilized in 50 mL of the electrolyte solution (0.1 mol L⁻¹ LiClO₄ in MeOH) poured into the anodic compartment and 50 mL of the electrolyte solution were added into the cathodic compartment. The voltage applied was 1.3 V *versus* Ag/AgCl (KCl 3 mol L⁻¹) during 8 h. The working and counter electrodes were cleaned properly every 30 min. The progress of the reaction was monitored by taking aliquots (40 µL) from the reaction mixture at different time intervals, which were analyzed by TLC. At the end, 25 mL of distilled H₂O were added to the anodic content. The reaction solution was extracted with EtOAc and the organic phase was washed with distilled H₂O. After dried with MgSO₄ and evaporation of the solvent under reduced pressure, 40 mg of brown oil was obtained. This material was purified using Sephadex LH-20 (30 × 2 cm), eluted with MeOH, to afford 18 mg of MOB. HPLC analysis performed to starting material (biseugenol) and to MOB indicated that the product was obtained in high purity (> 99%).

Methoxyl-oxo-biseugenol (MOB)

Amorphous solid; ¹H NMR (300 MHz, CDCl₃) δ 7.35 (d, 1H, *J* 1.8 Hz, H-6), 6.80 (d, 1H, *J* 2.1 Hz, H-2'), 6.59 (d, 1H, *J* 2.1 Hz, H-6'), 6.09-5.81 (m, 2H, H-8 and H-8'), 5.17-5.02 (m, 5H, H-2, H-9 and H-9'), 4.02 (s, 6H, 3-MeO and 3'-OMe), 3.97 (s, 3H, 3-OMe), 3.84 (d, 2H, *J* 6.5 Hz, H-7'), 3.59 (d, 2H, *J* 6.6 Hz, H-7); ¹³C NMR (75 MHz, CDCl₃) δ 191.7 (C-4), 159.4 (C-2), 157.4 (C-6), 137.3 (C-5'), 135.3 (C-8 and C-8'), 116.2 (C-9'), 115.9 (C-9), 113.8 (C-2'), 110.1 (C-3), 56.1 (3-MeO and 3'-OMe), 56.0 (3-OMe), 40.6 (C-7 and C-7'); HRMS (ESI) *m/z*, calcd. for C₂₁H₂₄O₅Na [M + Na]⁺: 379.1521, found: 379.1523; calcd. for C₄₂H₄₈O₁₀Na [2M + Na]⁺: 735.3145, found: 735.3146.

Cell viability

All cell lines (B16F10-Nex2, SKMEL-25, MØ Raw 264.7, and HUVEC) were cultivated in low glucose Roswell Park Memorial Institute (RPMI, Buffalo, NY, USA) supplemented with 2 g L⁻¹ sodium bicarbonate, 10% of fetal bovine serum (FBS), 59 µg mL⁻¹ penicillin, 133 µg mL⁻¹ streptomycin and 40 µg mL⁻¹ gentamicin at 37 °C in a CO₂ incubator. All cells (1 × 10⁴) in 100 µL of medium were added to 96 well plates. After five hours, the medium was replaced by fresh medium containing serial dilutions (200.0-0.4 mg mL⁻¹) of biseugenol, MOB and positive control cisplatin. The incubation proceeded for 24 h at 37 °C.

In all cases, 10 µL of PrestoBlue (Invitrogen, Carlsbad, CA, USA) were added and the plates with the cells were further incubated for four hours at 37 °C for the mammalian cells. Absorbance at 560 and 600 nm in each well were measured by using a GloMax Explorer (Promega Biotecnologia, São Paulo, SP, Brazil). The differences between absorbance at 560 and 600 nm for each well were normalized against a blank value for medium without cells and lysed cells. Data was analyzed using GraphPad Prism 5.0²⁸ with logarithmic nonlinear regression to generate a response fitting curve and determine the inhibitory concentration that affects 50% of cells (IC₅₀). Each cell line was tested in triplicates in up to three independent experiments. Selective index (SI) was estimated by the ratio between IC₅₀ values to murine cells MØ Raw 264.7 and B16F10-Nex2 or between the IC₅₀ values to human HUVEC and SKMEL-25 cells.²⁹

Results and Discussion

Biseugenol was prepared by reacting eugenol with potassium hexacyanoferrate(III) and its structure was confirmed by comparison of NMR (¹H and ¹³C) and ESI-HRMS data with those reported in the literature.^{3,27} First, the electrochemical behavior of biseugenol was examined in 0.1 mol L⁻¹ LiClO₄/MeOH (electrolyte solution) using cyclic voltammetry. The electrolyte has no redox peaks in the potential range from -1.0 to 1.25 V (*vs.* Ag/AgCl KCl 3 mol L⁻¹) (Figure 1, curve in black). After 1.25 V the oxidation of the electrolyte is observed (Figure 1 highlighted with a gray rectangle).

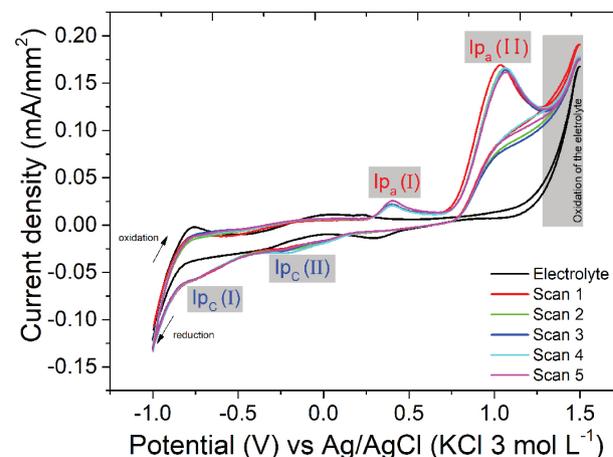


Figure 1. Cyclic voltammetry of the biseugenol (3 mmol L⁻¹) in LiClO₄/MeOH (0.1 mol L⁻¹) scan rate = 100 mV s⁻¹. Working electrode = Pt, counter electrode = Pt, reference electrode = Ag/AgCl (KCl 3 mol L⁻¹). Ip_a: oxidation peaks and Ip_c: reduction peaks.

The cyclic voltammograms of biseugenol are seen in Figure 1. In the first cycle (Figure 1, curve in red), biseugenol displayed an anodic peak at ca. 0.4 V *vs.* Ag/AgCl 3 mol L⁻¹

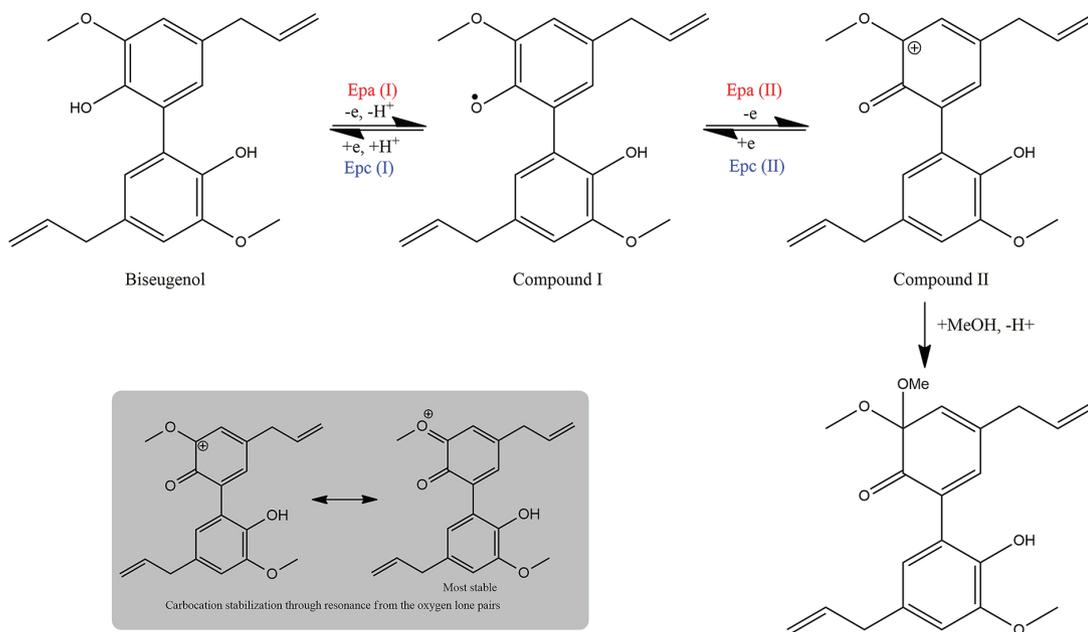


Figure 2. Proposed mechanism for electrochemical behavior of biseugenol.

(I_{p_a} I) and another around 1.05 V (I_{p_a} II) and a cathodic peak at ca. -0.25 V and finally the last one around -0.75 V. In concordance with the usual electrochemical behavior of phenol and other methoxyphenol derivatives,^{20,30-33} we proposed that biseugenol yields a phenoxy radical by giving one electron and one proton (compound I, Figure 2), followed by a second electron loss producing a cation (compound II, Figure 2), which is stabilized by the assistance of the oxygen of the methoxyl group (Figure 2). This carbocation is susceptible to further reaction with methanol that acts as nucleophile, consistent with the mechanism illustrated in Figure 2. This last process is an irreversible chemical reaction. In the reverse scan of the cycle, a small amount of the stabilized carbocation is reduced by accepting two electrons and one proton (I_{p_c} I and I_{p_c} II), affording the starting material (Figure 2). In the subsequent cycles (Figure 1, cycles 2 to 5) the same processes are detected.

Additional cyclic voltammetry experiments were conducted by varying the concentration of the analyte (Figure 3).

Both anodic and cathodic peak currents (I_{p_a} or I_{p_c}) increased linearly with the concentration of biseugenol, confirming that these redox processes are due to the oxidation and reduction of the analyte, respectively. In the inset of Figure 3 is seen that I_{p_a} II varies linearly with the analyte concentration. Besides, it was observed that the anodic peak currents shift to more positive potentials from lower to a higher concentration (indicated as dashed arrows in Figure 3). The reason for this behavior is that at a lower concentration of biseugenol the diffusion occurs more

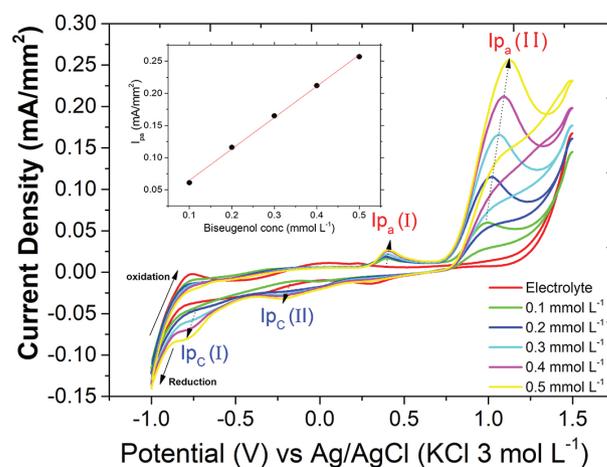


Figure 3. Cyclic voltammetry (first cycle) of the biseugenol varying concentration in $\text{LiClO}_4/\text{MeOH}$ (0.1 mol L^{-1}) scan rate = 100 mV s^{-1} . Working electrode = Pt, counter electrode = Pt, reference electrode = Ag/AgCl (KCl 3 mol L^{-1}).

easily than at a higher concentration. The same behavior is observed for the anodic peak currents, which are shifted for more negative potentials.

It is worth to mention that biseugenol is a symmetrical molecule, so it is logical to expect that both phenolic moieties oxidize simultaneously. However, it is already published in the literature³⁴ that the potentials of the redox processes for each phenolic moiety in the 2,2'-biphenol are different since the deprotonation that occurs during the electron transfer are distinct. The first pK_a is about 6 units of pK_a lower than the second pK_a . This pK_a splitting can be attributed to a stabilizing intramolecular hydrogen bond between $-\text{O}^-$ and $-\text{OH}$ in the monoprotonated 2,2'-biphenol, as shown in Figure 4. This stabilizing effect

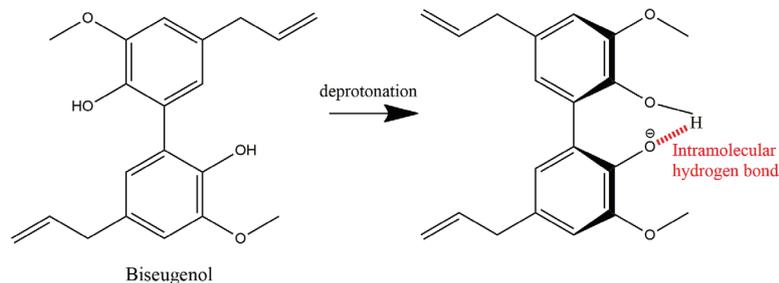


Figure 4. Stabilizing intramolecular hydrogen bond in the biseugenol after deprotonation.

is also reflected in the one-electron transfer potential of the corresponding phenoxy radical.

The exhaustive oxidation of the biseugenol was performed at 1.30 V to ensure the formation of the radical cation. This procedure was performed in a two-compartment electrochemical cell to prevent side reactions that occur at the auxiliary electrode affect either the starting materials or the products in the cathode compartment. The oxidation was carried out for 8 h and, every 30 min, the electrodes were meticulously cleaned to ensure that deposits on the cathode and anode surfaces were removed. The current *versus* time plots for each 30 min cycle are shown in the inset of Figure 5.

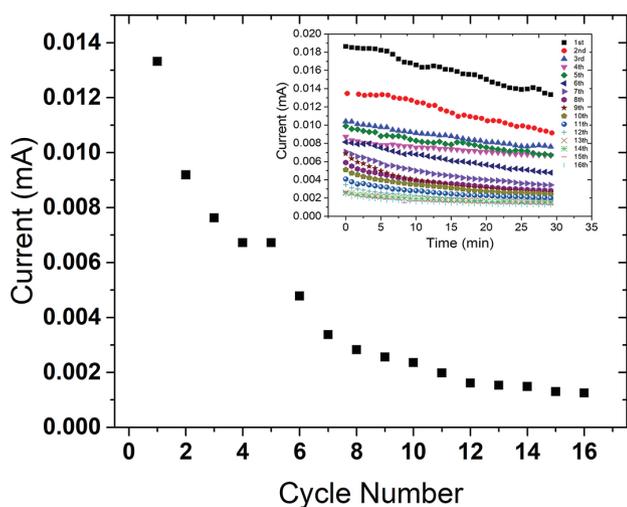


Figure 5. Current at the end of each cycle *versus* cycles during the electrolysis. Inset: current *versus* time plots for each 30 min cycle.

The curves have the same profile, in other words, the current decreases over time due to the consumption of the reagent. In the curve of the current at the end of each cycle in the function of cycle number (Figure 5) it is noted a pronounced drop until the 10th cycle and in the subsequent cycles, the current is practically constant. The progress of the reaction was monitored by TLC, collecting aliquots of the reaction mixture every 30 min. After the end of the reaction, the anode content was treated with H₂O and extracted using EtOAc. After drying and removal of the

organic solvent, the crude material was fractionated through a Sephadex LH-20 column using MeOH as eluent, to afford pure MOB.

¹H NMR spectrum (Figure S1, Supplementary Information section) of oxidized compound exhibited two doublets at δ 3.59 (2H, *J* 6.6 Hz, H-7)/3.84 (2H, *J* 6.5 Hz, H-7') which, in association to the multiplets assigned to H-8/H-8' at δ 6.09-5.81 (2H) and to H-9/H-9'/H-2 at δ 5.17-5.02 (5H), allowed the identification of allyl moieties, as observed in the biseugenol molecule. Aromatic hydrogens H-6', H-2', and H-6 were observed as doublets at δ 6.59 (1H, *J* 2.1 Hz), 6.80 (1H, *J* 2.1 Hz) and 7.35 (1H, *J* 1.8 Hz), respectively. The deshielding observed to H-6 suggested that hydroxyl group at C-4 in the structure of biseugenol was oxidized to carbonyl group. Furthermore, two singlets at δ 4.02 (6H) and 3.97 (3H) indicated, beside the methoxyl groups at C-3 and C-3' present in the structure of original biseugenol, an additional methoxyl group at C-3, similarly to that observed in the oxidation of eugenol.^{20,30,35}

The occurrence of a conjugated carbonyl group was confirmed by analysis of ¹³C NMR spectrum (Figure S2, Supplementary Information section), which showed, besides other signals, the carbonyl group at δ 191.7 (C-4), sp² carbons ranging from δ 115.9-159.4, methylene group of allyl groups (C-7 and C-7') at δ 40.6 and methoxyl groups at δ 56.1 and 56.0. Finally, ESI-HRMS (Figure S3, Supplementary Information section) showed [M + Na]⁺ and [2M + Na]⁺ ion-peaks at *m/z* 379.1523 and 735.3146, respectively, in accordance with molecular formula C₂₁H₂₄O₅.

Therefore, the structure of MOB, a new derivative from biseugenol was determined as indicated in Figure 6. HPLC analysis performed to starting material (biseugenol) and to the new compound (MOB) showed that the product was obtained in high purity (>99%) (Figure S4, Supplementary Information section).

Considering the anticancer activity of eugenol,³⁶⁻³⁸ the cytotoxic potential of biseugenol and its oxidized derivative MOB was evaluated against tumoral cells B16F10-Nex2 (murine metastatic melanoma) and SKMEL-25 (human

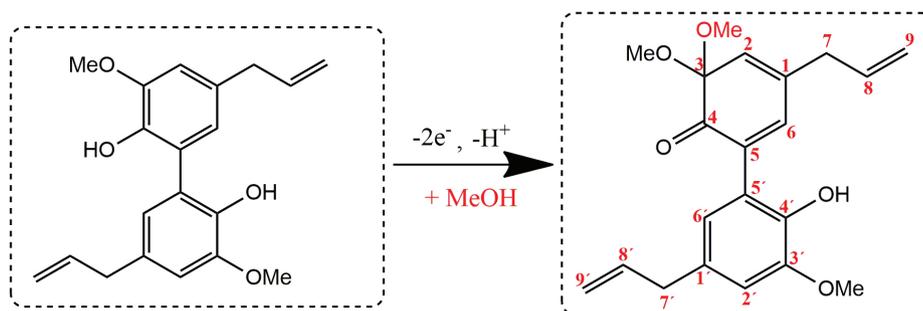


Figure 6. Oxidation of biseugenol in MeOH to form methoxyl-oxo-biseugenol.

Table 1. Values of 50% inhibitory concentration (IC_{50}) of biseugenol, methoxyl-oxo-biseugenol (MOB) and positive control cisplatin against murine B16F10-Nex2 and human SKMEL-25 melanoma cells and non-tumoral lines from murine (MØ Raw 264.7) and human (HUVECs) background

| Cell line | IC_{50} / ($\mu\text{g mL}^{-1}$) | | | SI ^a | | |
|--------------|---------------------------------------|------------|------------|-----------------|-----|-----------|
| | Biseugenol | MOB | Cisplatin | Biseugenol | MOB | Cisplatin |
| B16F10-Nex2 | 21.4 ± 6.7 | 9.5 ± 1.6 | 53.1 ± 4.2 | 1.4 | 2.1 | 1.1 |
| SKMEL-25 | 34.2 ± 3.1 | 13.2 ± 2.5 | > 200 | 1.7 | 2.8 | – |
| MØ Raw 264.7 | 29.3 ± 1.9 | 19.9 ± 3.5 | 56.2 ± 5.5 | – | – | – |
| HUVEC | 57.8 ± 8.8 | 36.3 ± 7.4 | 7.3 ± 0.4 | – | – | – |

^aSI (selectivity index) = IC_{50} MØ Raw 264.7/ IC_{50} B16F10-Nex2 or IC_{50} HUVEC/ IC_{50} SKMEL-25. IC_{50} : 50% inhibitory concentration; B16F10-Nex2: murine metastatic melanoma; SKMEL-25: human metastatic melanoma; MØ Raw 264.7: murine macrophage immortalized; HUVEC: human umbilical endothelium.

metastatic melanoma) as well as against non-tumoral cells MØ Raw 264.7 (murine macrophage immortalized) and HUVEC (human umbilical endothelium), in order to calculate the respective selectivity index (SI). As observed in the obtained data as indicated in Table 1, biseugenol killed 100% of cytotoxic cells at the highest tested concentration, resulting in IC_{50} values of 21.4 ± 6.7 and $34.2 \pm 3.1 \mu\text{g mL}^{-1}$ for B16F10Nex2 and SKMEL-25 cells, respectively, indicating the superior potential of positive control cisplatin.

Furthermore, these compounds also displayed activity against non-tumoral cells MØ Raw 264.7 and HUVEC, with IC_{50} values of $29.3 \pm 1.9 \text{ mg mL}^{-1}$ (SI of 1.4) and $57.8 \pm 8.8 \text{ mg mL}^{-1}$ (SI of 1.7), respectively. Considering the IC_{50} values of MOB, determined as 9.5 ± 1.6 and $13.2 \pm 2.5 \text{ mg mL}^{-1}$ for B16F10Nex2 and SKMEL-25 cells, respectively, it is possible to conclude that the oxidized derivative MOB exhibited higher activity than biseugenol which could be partially explained by the presence of a non-aromatic ring and a conjugated carbonyl system instead of a phenolic moiety. This compound also showed activity against non-tumoral cells MØ Raw 264.7 and HUVEC, with IC_{50} values of $19.9 \pm 3.5 \text{ mg mL}^{-1}$ (SI of 2.1) and $36.3 \pm 7.4 \text{ mg mL}^{-1}$ (SI of 2.8), respectively. As observed, the SI values to MOB were superior to those determined to biseugenol suggesting that this compound could be used as a future scaffold to development of new bioactive derivatives based on the structure of natural product eugenol and biseugenol.

Conclusions

Biseugenol undergoes two-electron oxidation under controlled potential producing a cation, which is stabilized by the assistance of the oxygen of the methoxy group, followed by chemical reaction with MeOH, used as an electrolyte. The procedure afforded a new oxidized derivative, MOB, which contains a non-aromatic ring and a conjugated carbonyl system instead of a phenolic moiety, as observed in the reagent structure. Due to this feature, MOB exhibited higher anticancer activity than biseugenol. Therefore, since this synthetic procedure is clean, many other natural products can be transformed by electrochemical procedures.

Supplementary Information

Supplementary information is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

Acknowledgments

This work was supported by grants from São Paulo State Research Foundation (FAPESP 2018/07885-1 and 2018/20826-4). We also thank CNPq for the grant award to João Henrique G. Lago, Fernanda F. Camilo and Luiz R. Travassos. Flavia T. da Silva is also thankful to CAPES for the provided scholarship (finance code 001).

Author Contributions

Fernanda F. Camilo, João Henrique G. Lago, Tereza S. Martins and Luiz R. Travassos designed the research and provided guidance; Flavia T. da Silva, Kaio S. Gomes, Fabricio C. Machado and Leticia L. Loureiro performed the experiments; João Henrique G. Lago and Fernanda F. Camilo wrote the manuscript. All authors read and approved the final manuscript.

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Submitted: September 25, 2020

Published online: January 6, 2021

