Antioxidant Activity of New Carvone Derivatives

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Abstract

Carvone is one of the most versatile synthons in organic synthesis. It has been used as starting material for a huge number of organic syntheses of natural products with interesting biological activities. This manuscript communicates the antioxidant activity against superoxide of carvone and several derivatives.

Keywords
carvone, antioxidant activity, superoxide ion, synthesis, natural products

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The importance of natural products in pharmacy and medicine as a source of bioactive compounds is out of doubt. Our group has tried to use different natural products such as sclareol1-3 or ent-halic acid4-6 as starting material for the synthesis of compounds with biological interest. In the last years, we have been involved in the synthesis of carvone derivatives that could be used as building blocks in organic chemistry as 2, see Figure 1,7 or as antioxidants as (R)-carvone 1 and its chloro-derivative 3.8 Several diseases are associated with oxidative stress caused by free radicals.9 Recently, we have communicated the synthesis of other carvone derivatives obtained for the first time, 4, 5, 7, and 8, using organocatalysis.10 In this work, we report the synthesis of (R)-carvone derivatives 6, 9, 10 and the study of their antioxidant activity as well as for other related compounds.

The structure of compounds 6, 9, and 10 was determined studying the 1D and 2D NMR, IR, and HRMS taking into account the data shown in the experimental section.

The Nuclear Magnetic Resonance (NMR) data are shown in Tables 1–3 for the carvone derivatives 6, 9, and 10. Compounds 4, 5, 7, and 8 have been described previously by us.6

2,2-Diphenyl-1-Picrylhydrazil Radical (DPPH•) Scavenging Activity

To assess the in vitro antioxidant activity, we first examined whether compounds possessed antiradical activity using 2,2-diphenyl-1-picrylhydrazil radical (DPPH•) scavenging assay.

2,2-Diphenyl-1-picrylhydrazyl radical is not a radical with biological importance, however, this assay is considered a valid accurate, easy, and economic method to evaluate radical scavenging activity of antioxidants, since the radical compound is stable and it has not been generated.11

In this model, we employed quercetin and curcumin as standards, which have shown a significant antiradical ability against DPPH, with the maximum inhibitory effect of 96.8% ± 0.1% and 98.7% ± 1.8%, respectively (Figure 2).

The evaluated compounds showed variable scavenging activities for this free radical. So, in Figure 2(a), in which we collected a group of evaluated monoterpenes, we observed that these compounds had no significant antiradical activity when we compared them with positive controls. Activities obtained with these terpenes were between 20% and 30%.

The compounds that presented the higher antiradical activity against DPPH were 5, 6, 9, and 10 which showed a catchment percentage of 47.6 ± 3.9, 54.8 ± 2.2, 61.6 ± 8.0, and 57.4 ± 3.2, respectively, with this effect being observed at the maximum tested concentration (Figure 2(b)).
Nitric Oxide Radical Scavenging Activity

Nitric oxide radical (NO•) plays an important role in the control of various physiological and pathophysiological pathways. Although NO in low concentrations is not toxic, during pathological conditions, it can become harmful due to its high reactivity with other free radicals, such as superoxide anion (O2•−). In anaerobic conditions, the NO is highly unstable and reacts with the oxygen to produce intermediate products (NO2, N2O4, and N3O4). In this condition, the NO could react with the O2•− and generate peroxynitrite (ONOO−), a strong oxidant that reacts with most biological molecules, causing cell damage.

This delicate balance between the physiological and the pathological role must be achieved through the inhibition of excess NO. It can also be achieved by reducing O2•− levels, thus preventing the production of reactive nitrogen species (RNS).

Table 1. ¹H and ¹³C NMR, Correlation HSQC and HMBC for Compound 6.

| C    | δ (ppm) | ¹H (ppm) | HMBC |
|------|---------|----------|------|
| 1    | 199.4   | -        | -    |
| 2    | 135.1   | -        | -    |
| 3    | 145.2   | 6.74 (1H, m) | 1, 4, 5, 10 |
| 4    | 26.2    | 2.22 (1H, d, J = 16.0, 14.1 Hz) | 5, 7 |
|      |         | 2.39 (1H, m) | 2, 3, 5 |
| 5    | 40.9    | 2.49 (1H, m) | 4, 6, 7 |
| 6    | 39.1    | 2.22 (1H, d, J = 16.0, 14.1 Hz) | 1, 4, 5, 7 |
|      |         | 2.39 (1H, m) | 1, 2, 4, 5 |
| 7    | 76.0    | -        | -    |
| 8    | 36.6    | 3.40 (1H, d, J = 11.1 Hz) | 5, 7, 9 |
|      |         | 3.44 (1H, d, J = 11.1 Hz) | 5, 7, 9 |
| 9    | 18.1    | 1.23 (3H, s) | 5, 7, 8 |
| 10   | 15.6    | 1.74 (3H, s) | 1, 2, 3 |
| CH3O-| 49.7    | 3.21 (3H, s) | 7    |

Table 2. ¹H and ¹³C NMR, Correlation HSQC and HMBC for Compound 9.

| C    | δ (ppm) | ¹H (ppm) | HMBC |
|------|---------|----------|------|
| 1    | 190.7   | -        | -    |
| 2    | 132.7   | -        | -    |
| 3    | 142.6   | 6.55 (1H, d, J = 4.3, 1.4 Hz) | 1, 4, 5, 10 |
| 4    | 22.6    | 2.52 (1H, d, J = 20.5, 6.8, 2.8 Hz) | 2, 3, 6, 7 |
|      |         | 2.92 (1H, d, J = 20.5, 6.8, 2.8 Hz) | 6    |
| 5    | 48.3    | 3.15 (1H, m) | 1, 3, 6, 7, 9 |
| 6    | 46.8    | 4.51 (1H, t, J = 1.7 Hz) | 1, 2, 4, 7 |
| 7    | 86.1    | -        | -    |
| 8    | 35.0    | 4.02 (1H, d, J = 11.2 Hz) | 5, 7, 9 |
|      |         | 4.15 (1H, d, J = 11.2 Hz) | 5, 7, 9 |
| 9    | 20.8    | 1.78 (3H, s) | 5, 7, 8 |
| 10   | 15.5    | 1.52 (3H, s) | 1, 2, 3 |
| 1′   | 164.1   | -        | -    |
| 2′   | 132.7   | -        | -    |
| 3′   | 164.6   | -        | -    |
| 4′   | 123.9   | 8.03 (1H, d, J = 7.8, 1.2 Hz) | 3′, 6′ |
| 5′   | 131.1   | 7.63 (1H, td, J = 7.8, 1.6 Hz) | 3′, 7′ |
| 6′   | 133.6   | 7.71 (1H, td, J = 7.5, 1.2 Hz) | 4′, 7′ |
| 7′   | 128.9   | 7.57 (1H, dd, J = 7.5, 1.5 Hz) | 1′, 3′, 5′ |

The model of antiradical activity against NO applied in this assay was an indirect method to predicting the ability of a compound to capture the excess of NO, and in this way, we prevent the generation of ONOO−.

Table 4 shows the results obtained when the assayed compounds were evaluated against the NO radical. The percentage of NO scavenger developed by the pattern compounds quercetin and curcumin was 31.3 ± 2.5 and 29.2 ± 2.0, respectively, which could be considered as a discrete activity. For quercetin, the maximum activity was registered by the concentration of 1 × 10⁻⁸ M, while for curcumin the maximum inhibitory effect was observed with the 1 × 10⁻⁹ M.

Same as the pattern compounds, the activity of all terpenes to capture the NO was lowered to 50%. We observed that the
mixture 3 + 4 developed a scavenging activity of 43%, which was significantly higher than the value obtained for quercetin and curcumin and more than the activity shown by 3 and 4 separately. This difference could be explained by a synergistic effect. It is noticeable that just 2 terpenes, 5 and 6 which exhibited a good activity in DPPH model, in NO assay showed similar behavior to reference substances (Table 4). Additionally, in this model, compound 8 developed a 34.4% inhibition of the nitric oxide radical. It should be noted that the activity developed by the compounds did not show a concentration-response relationship; therefore, for obtaining IC<sub>50</sub>, we practiced an estimated analysis using the program Graphpad prism v.5 test Equation: log(inhibitor) vs normalized response—Variable slope (Table 4). From the estimates obtained, we can observe that the potency does not vary significantly between the effective terpenes and the patterns.

**Assay of Superoxide Radical Scavenging Activity**

Superoxide anion is one of the free radicals of higher biological importance since it is in greater concentration on the organism, with the initiator of oxidative stress chain, constituting the main precursor of other reactive species of oxygen and nitrogen.

In the model tested, O$_2^-$ was produced chemically without the presence of enzymes (nonenzymatic model) which determines the percentage of superoxide anion uptake. Thus, in this system, the evaluated compounds can exert an antioxidant activity by reducing the production of O$_2^-$, or by a stabilizing action of the radical when donating or receiving electrons to the O$_2^-$ radical.

In Table 4, the results obtained by the evaluation of set of compounds are shown. The monoterpenes that were derived from Carvone, 4, which was a halogen monoterpene, showed a maximum inhibitory effect of 35.0% ± 1.0% and this maximum scavenger effect did not show differences with the quercetin control (31.3% ± 0.6%). For the monoterpenes Carvone 1 (12.2% ± 1.0%), 3 (16.2% ± 1.3%), mixture 3 + 4 (15.0% ± 0.8%), 6 (12.9% ± 1.6%), and 10 (15.9% ± 0.7%), the pickup activity of the radical was more similar to that obtained with curcumin (13.1 ± 0.8%). Rest of the monoterpenes did not evidence significant antiradical effects against O$_2^-$.

**Vascular Superoxide Production Assay**

The antioxidant activity evaluation against O$_2^-$ in the presence of lucigenin is achieved by the chemoluminescence measurement of the formation of O$_2^-$ in a biological medium as the aorta rings. It was determined by taking into account that the diminution of the chemoluminescence signal indicates less O$_2^-$ concentration, therefore a higher antioxidant activity. This system permits the evaluation of another mechanism of activity antioxidant in an indirect way as the possible modulation of pro-oxidants’ enzymatic systems. It has been demonstrated that the incubation of the rings with the inhibitors of NADPH oxidase, nitric oxide synthase, xanthine oxidase, and the cyclooxygenase stops the anion superoxide production and lucigenin is not able to generate a good signal. Although lucigenin can potentiate that production of the superoxide anion radical without the pro-oxidative enzymes previous action in the production of that radical, lucigenin, it is not able to generate superoxide anion de novo.

Our results show that the baseline production of O$_2^-$ in aortic rings incubated with lucigenin was significantly inhibited in the presence of the reference substances quercetin and curcumin, whose relative luminescence unit (RLU) values at maximum concentration were 223.3 ± 3.3 and 261.1 ± 43.1, respectively (Figure 3; Table 4). At the same concentration, monoterpenes 1 and mixture (3 + 4) developed the same inhibitory capacity as the patterns (201.7 ± 12.0 and 210.2 ± 16.3).
This result is related to those obtained in the NO inhibition model, where the mixture \( 3 + 4 \) proved to be more effective than the individual compounds.

In Figure 3, we can observe that, although to a lesser extent than compounds 1 and mixture \( 3 + 4 \), all the terpenes assayed are capable of inhibiting \( \text{O}_2 \cdot \) in a concentration-dependent manner. The maximum inhibitory capacity reached by both the terpenes and the reference compounds was observed when the rings were incubated with \( 1 \times 10^{-5} \) M.

The antioxidant efficacy of the compounds evaluated does not present a pattern, but, like the reference compounds, differs depending on the radical to be inhibited. It should be noted that despite the variability in the antioxidant activities developed by monoterpenes in different trials, it is the chemoluminescence model that offers the most reliable data. It is acknowledged that chemoluminescence can be very sensitive in the detection of low-level reactions because it provides a detectable response below the detection limit of most chemical assays.

If we think the structure activity relationship can be deduced for more substituted monoterpenes, the activity diminished in a biological media with the halogenated ones being most active.

### Conclusion

Three new compounds have been synthesized from (R)-carvone 1 and its derivatives. Radical scavenging activity has been determined by different methods, showing variable and interesting antioxidant activity.

### Experimental

#### General Methods

Unless otherwise stated, all chemicals were purchased with the highest purity commercially available and were used without further purification. Infrared (IR) spectra were performed on a
Genesis II ATR spectrophotometer. The samples were placed on the diamond, previously cleaned with isopropanol. The spectra were performed using the OMNIC software. $^1$H and $^13$C NMR spectra were performed in CDCl$_3$ and referenced to the residual peak of CHCl$_3$ at $\delta$ 7.26 ppm and $\delta$ 77.2 ppm, for $^1$H and $^{13}$C, respectively, using a Bruker Avant 400, 400 and 100 MHz spectrophotometer. Chemical shifts ($\delta$) are reported in parts per million (ppm) and coupling constants ($J$) are given in Hertz (Hz). Mass spectra are presented as $m/z$ (% rel. int.). High Resolution Mass Spectroscopy (HRMS) were recorded on a VG Platform (Fisons) spectrometer using chemical ionization (ammonia as gas) or fast atom bombardment technique. For some of the samples, QSTAR XL spectrometer was employed for electrospray ionization. The optical activity was determined on a Bellingham + Stanley Ltd. ADP 222 polarimeter, with 5 mL cells in chloroform solution. The concentration and temperature varied from compound to compound and were specified in the presentation of the values of the optical activity.

**Synthesis of Compound 6**

In a round-bottom flask equipped with a reflux condenser, (R)-carvone 1 (1.0 g, 6.67 mmol), N-Bromosuccinimide (NBS) (1.66 g, 9.33 mmol), and 20 mL of CH$_2$Cl$_2$ were heated at 39°C while stirring. The progress of the reaction was monitored by Thin Layer Chromatography (TLC). After 6 days, the reaction was extracted with CH$_2$Cl$_2$, washed with water, dried over anhydrous Na$_2$SO$_4$, and the solvent evaporated. Finally, the pure compound was obtained by flash chromatography on silica gel, hexane/EtOAc (80:20) (Table 2). Compounds 4 and 5 have been described previously by us.$^6$

Compound 6 was purified by flash chromatography (silica gel, hexane/EtOAc 80:20).

$$\alpha_{20}^D = +3.9 \text{ (c = 2.5 mg/mL CHCl}_3\text{). IR (film): 2976, 2829, 1645, 1530, 1450, 1348, 1288, 1248, 1101, 1081, 1064, 904, 745, 630 \text{ cm}^{-1}; HRMS (EI) calc. for C$_{17}$H$_{18}$BrNO$_5$Na requires (M+Na) 476.1306; found 476.1302. NMR data, see Table 3.}$^6$

**Synthesis of Compound 9**

In a round-bottom flask equipped with a reflux condenser, (R)-carvone 1 (1.0 g, 6.66 mmol), 2-nitrobenzoic acid (1.11 g, 6.66 mmol), and 30 mL of CH$_2$Cl$_2$ were heated at 39°C while stirring. The progress of the reaction was monitored by TLC. After 3 days, the reaction was extracted with CH$_2$Cl$_2$, washed with water, dried over anhydrous Na$_2$SO$_4$, and the solvent evaporated. Finally, the pure compound was purified by flash chromatography (silica gel, hexane/EtOAc 70:30).

$$[\alpha]_{20}^D = -8.30 \text{ (c = 1.5 mg/mL CHCl}_3\text{). IR (film): 3231, 2976, 2829, 1645, 1530, 1450, 1348, 1288, 1248, 1101, 1081, 1064, 904, 745, 630 \text{ cm}^{-1}; HRMS (EI) calc. for C$_{17}$H$_{18}$BrNO$_5$Na requires (M+H) 476.1306; found 476.1302. NMR data, see Table 2.}$^6$

**Synthesis of Compound 10**

In a round-bottom flask equipped with a reflux condenser, (R)-carvone 1 (1.00 g, 6.67 mmol), quinine 2% (0.046 g, 0.13 mmol), 2-nitrobenzoic acid (1.56 g, 9.33 mmol), NBS (1.66 g, 9.33 mmol), and 20 mL of CH$_2$Cl$_2$ were kept at room temperature while stirring. The progress of the reaction was monitored by TLC. After 6 days, the reaction was extracted with CH$_2$Cl$_2$, washed with water, dried over anhydrous Na$_2$SO$_4$, and the solvent evaporated. Finally, the pure compound was obtained by flash chromatography on silica gel (hexane/EtOAc) to obtain compound 10, 109.4 mg (4.3%), see Table 1.

Compound 10 was purified by flash chromatography (silica gel, hexane/EtOAc 90:10).

$$\text{IR (film): 3499, 2829, 1645, 1597, 1385, 1373, 1271, 1141, 1010, 904, 745, 630 \text{ cm}^{-1}; NMR data, see Table 3.}$^6$

2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH$^*$) Scavenging Activity

The DPPH free radical scavenging activity was estimated by assay based on the method described in the literature.$^{13}$ All compounds were dissolved in dimethylsulfoxide (DMSO) at different concentration solutions (1 × 10$^{-9}$-1 × 10$^{-4}$ M). Quercetin and curcumin were used as positive controls. The reaction mixtures contained 100 µL of sample solution and 100 µL DPPH methanol solution (0.3 mM), and then, the absorbance was measured at 515 nm after incubation at 37°C for 30 minutes. The DPPH radical scavenging effect was calculated using the following formula:

$$\% \text{ of free radical scavenging activity} = \left( {A_0 - A_1} \right) \times 100 \quad (1)$$

where $A_0$ is the absorbance of the DPPH instead of the sample and $A_1$ is the absorbance of sample.

Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging activity was estimated by assay based on Griess Illosvoy reaction.$^{14}$ About 50 µL of the test sample (1 × 10$^{-9}$-1 × 10$^{-4}$ M in DMSO) was mixed with 50 µL of sodium nitroprusside (10 mM), and 50 µL of Griess reagent prepared in saline phosphate buffer (pH 7.4). The microplates were incubated for 150 minutes at room temperature and the absorbance was recorded at 546 nm. Curcumin was used as a standard. The percentage of NO$^*$ radical scavenging was calculated according to Eq. (1).
Superoxide Radical Scavenging Activity

Measurement of O$_2^-$ scavenging activity of terpene compounds was based on the method described by Lin and Chou. Superoxide radicals were generated in a Phenazine Methosulfate - Reduced Nicotinamide Adenine Dinucleotide (PMS-NADH) system by the oxidation of NADH and assayed through the reduction of NBT. The superoxide radicals were generated with 50 µL of different concentrations of the sample (1 × 10⁻⁹-1 × 10⁻⁴ M), 50 µL of NBT (150 µM) solution, and 50 µL of NADH (936 µM) solution. The reaction started by adding 50 µL of PMS solution (120 µM) to the mixture, it was incubated at 25°C for 5 minutes, and the absorbance at 560 nm was then measured by an automated microplate reader (GloMax-Multi + Detection System with Instinct Software). Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage of superoxide radical scavenging was calculated according to Eq. (1).

Vascular Superoxide Production Assay

Vascular O$_2^-$ production was measured in thoracic aorta segments using lucigenin-enhanced chemiluminescence, as previously described. Vessels were incubated in a (-)-hydroxyethyl (1-piperazineethanesulfonic acid) HEPES buffer (in mM: NaCl, 119; HEPES, 20; MgSO$_4$, 1; KCl, 4.6; KH$_2$PO$_4$, 0.4; Na$_2$HPO$_4$, 0.15; NaHCO$_3$, 5; CaCl$_2$, 1.2; glucose, 5.5; pH 7.4) aerated (95% O$_2$/5% CO$_2$) and maintained at 37°C for 30 minutes. After an equilibrated period, samples were then transferred into fluorocence microplates containing 200 µL of the HEPES buffer, 5 µM lucigenin, and supplemented with the corresponding tested samples, positive controls (10⁻⁷ - 10⁻⁵ M) and the solvent DMSO. Under these conditions, superoxide levels were measured by chemiluminescence using a luminometer (Glomax Multi Detection System). Luminescence units were recorded every 30 seconds for 10 minutes. The relative values of O$_2^-$ production were expressed as RLU per milligram and minute of dry tissue (RLU/(mg·min)).

Statistical Analysis

Results of antioxidant activity are presented as mean ± standard deviation (mean ± SD). Minimum of 3 independent experiments were carried out. Statistical analysis was performed using one-way analysis of variance (one-way ANOVA) and significance difference between the treatments was accepted at the level of $P < 0.05$.

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