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Simple colorimetric method to determine the *in vitro* antioxidant activity of different monoterpenes

Nehuen Noacco[a], Boris Rodenak-Kladniew[b], Margarita García de Bravo[b], Guillermo R. Castro[a], German A. Islan[a,*]

[a] Laboratorio de Nanobiomateriales, CINDEFI, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata-CONICET (CCT La Plata), Calle 47 y 115, C.P. 1900 La Plata, Argentina
[b] Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET-UNLP, CCT-La Plata, Facultad de Ciencias Médicas, C.P. 1900 La Plata, Argentina

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ABSTRACT

The development of simple, fast and reproducible techniques that provide information about the antioxidant activity (AA) of different compounds is essential to screen and discover new molecules with potential applications in the therapeutic, cosmetic, toxicological and food fields. Here, a novel and simple colorimetric method ("*BCB assay*") is proposed for measuring the AA of chemical compounds by protection of the reporter dye Brilliant Cresyl Blue (BCB) from loss of color due to oxidation by hypochlorite (a physiological oxidant). The decay in BCB blue color (λ_{max} = 634 nm) in the presence of hypochlorite occurred in only 5 min and was used to track the AA of different molecules. Particularly, the AA of monoterpenes was demonstrated and used to quantify them at milimolar concentrations. Natural antioxidants like vitamins C and E, resveratrol, dithiothreitol, *N*-acetyl-L-cysteine and glutathione were used as controls to validate the assay. Linalool, geraniol and 1,8-cineole were tested and showed *in vitro* AA in a concentration-dependent manner. The monoterpene concentrations providing 50% protection against oxidation (AA_{50}) were 2.3, 36.2 and 135.0 mM for linalool, geraniol and 1,8-cineole respectively, suggesting interesting AA. The method provides a useful, fast, simple and low-cost tool to determine the *in vitro* AA of different molecules.

Introduction

Oxidative stress in biological systems is a complex process characterized by an imbalance between the production of reactive oxygen species (ROS, free radicals) and the metabolic ability to eliminate them [1]. A wide range of toxic reactions are produced in the tissues, mainly mediated by the presence of ROS species or precursors such as hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH^{\cdot}$), singlet oxygen ($^1O_2$), and superoxide anion radical ($O_2^{\cdot-}$), among others. Different pathologies, ranging from myocardial and neurological degeneration to cancer initiation, are commonly associated with oxidative stress [2]. In general, biological mechanisms involving detox enzymes (*i.e.*, superoxide dismutase, catalase) are triggered to prevent oxidative reactions. Besides, several nonenzymatic antioxidants play a key role in the maintenance of homeostasis. In this group, β-carotene, glutathione, melatonin and vitamins C and E are important protagonists [3].

A rapid detection of the antioxidant properties of biological molecules is an interesting tool to screen and discover new applications in the therapeutic, cosmetic and food fields. Although many techniques to detect AA were reported in the literature (*i.e.*, TBARS), no one included simple and cheap protocols and most of them may lack acceptable reproducibility [4]. In this sense, the determination of antioxidant activities, especially for molecules present in complex multiphase systems or having many biological functions, must be evaluated satisfactorily by several test procedures to discard the variables influencing the results [5].

There is a great diversity of reported methods to determine the antioxidant activity of phytomolecules, including flavonoids and phenolic compounds, which can be classified into two large groups: those that measure lipid peroxidation (TBARS, MA/HPLC, MA/GC, b-carotene assays) and those based on electron or radical scavenging (DPPH, ABTS, FRAP, FTC, etc.) [6]. Although lipid peroxidation-based assays are commonly used, especially TBARS, they present some limitations associated with their cost (since they require the use of several reagents), time (the reactions are carried out in different stages that involve heating, evaporation of solvents and the like) and/or the need for sophisticated equipment (HPLC, GC devices, specific columns). On the other hand, among the radical scavenging methods, the two most

* Corresponding author.
E-mail address: germanislan@biol.unlp.edu.ar (G.A. Islan).

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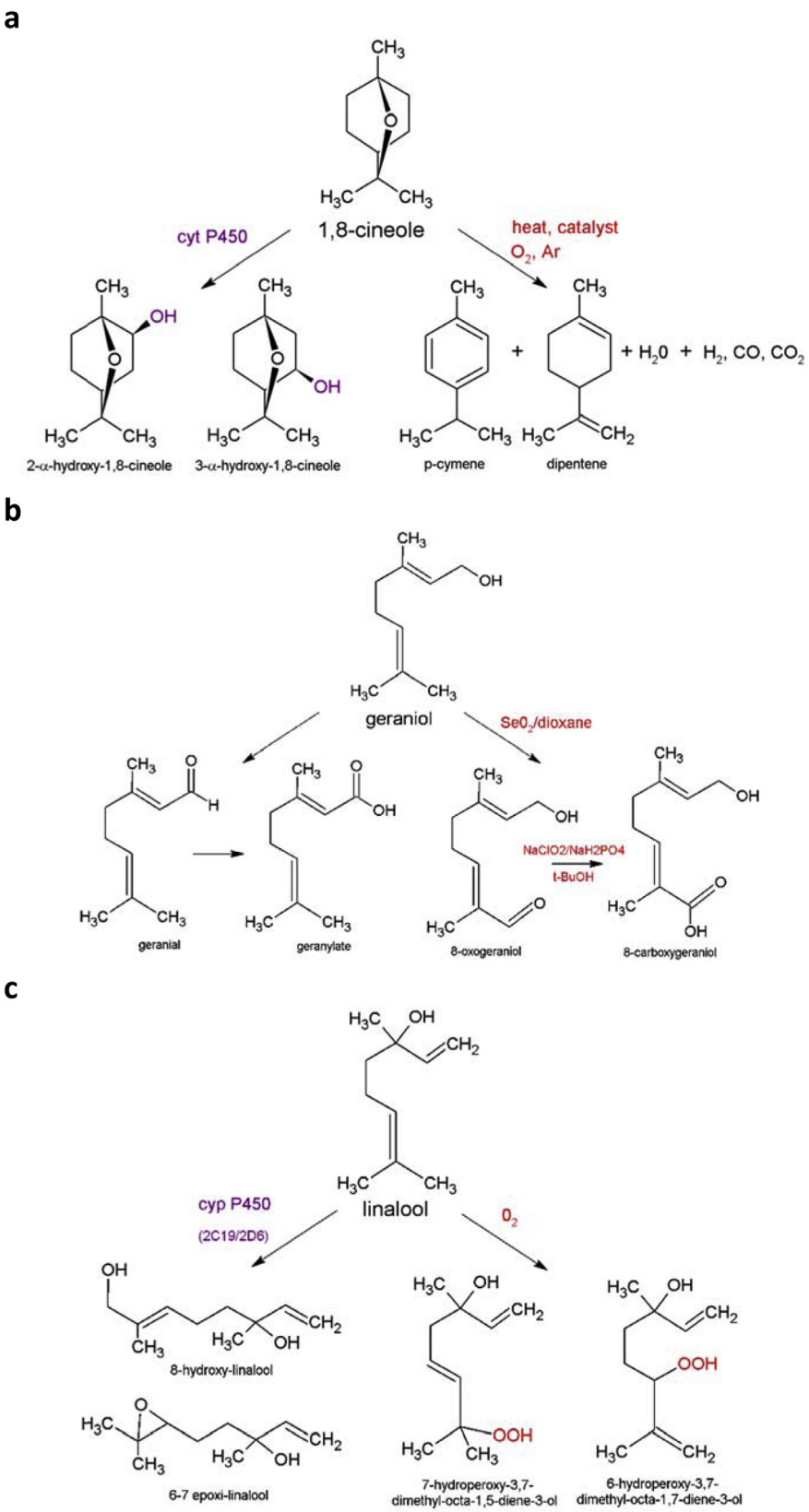

Fig. 1. Scheme of the possible oxidation products of 1,8-cineole (a), geraniol (b) and linalool (c) generated by different oxidizing agents.

widely reported are the DPPH and ABTS, since they are considered simple, sensitive, relatively fast and easily measurable methods (UV-Vis). Some limitations of these methods involve the use of non-physiological radicals, the need to previously generate these molecules ($ABTS^{\bullet+}$), compounds which spectrum is overlapping DPPH readment (i.e., carotenoids), relatively small linear reaction ranges and anti-oxidant reactions limited by steric accessibility to the radical, among others [7].

Antioxidants can be defined as molecules that are able to reduce or inactivate ROS, and different potential candidates can be found in nature. Among them, monoterpenes such as 1,8-cineole, geraniol and linalool appear as interesting alternatives to be explored [8]. The chemical structure of monoterpenes provides double bonds and reduced functional groups that are susceptible to oxidation producing different chemical species as is shown in Fig. 1 [9–15]. There are few precedents in the literature for the measurement of the antioxidant activity of monoterpenes, and most of them describe the use of DPPH as a suitable technique [16–18]. In the case of DPPH and ABTS assays, both use nonphysiological radicals as substrates, which are not present in biological systems [19].

On the other hand, common assays to detect monoterpenes involve the use of chromatographic techniques (usually gas chromatography with mass spectroscopy) which are expensive and time-consuming [20,21]. In this sense, the development of new techniques that provide a rapid detection of monoterpenes (particularly those that cannot be detected by UV-Vis, such as 1,8-cineole) represents a real need in the analytical field.

Among the group of dyes that could be used as reporters, Brilliant Cresyl Blue (BCB) is an oxazine commonly used as supravital stain with many biological applications such as reticulocyte counting or hemoglobin H precipitation. Besides, BCB shows a blue color in solution that turns into violet when it is exposed to oxidizing agents (Fig. 2). The dye was selected considering its susceptibility to be oxidized by chemical agents, the difference in the spectrum profile between the oxidized/reduced state, and due to its low toxicity [22].

The aim of the present work is the development of a fast and easy method to compare the antioxidant properties of different monoterpenes using BCB as reporter dye. In addition, the method provides linear dose/response ranges that could be useful to quantify this kind of compound. The ability of monoterpenes to prevent BCB oxidation by sodium hypochlorite was tested spectrophotometrically following the decay of the blue color of the dye. The method was validated with well-known antioxidants such as Vitamin C, dithiothreitol, *N*-acetyl-L-cisteine, L-glutathione and resveratrol as standards [23].

Materials and methods

Materials

Brilliant Cresyl Blue (phenoxazin-5-ium, 1,3-diamino-7-(diethylamino)-8-methyl-chloride, MW = 385.9 g/mol) was purchased from GURR® (*Hopkin* and *Williams* Ltd., batch No. 006120, London, UK). Linalool (MW = 154.25 g/mol, purity > 95%), 1,8-cineole (MW = 154.25 g/mol, purity = 99%), geraniol (MW = 154.25 g/mol, purity = 98%), α-tocopherol (Vitamin E, MW = 430.71 g/mol), resveratrol (MW = 228.25 g/mol, purity ≥ 99%), 1,4-dithiothreitol (MW = 154.25 g/mol, purity > 97%), *N*-acetyl-L-cisteine (MW = 163.19 g/mol, purity ≥ 99%), L-glutathione reduced (MW = 307.32 g/mol, purity ≥ 98%) and L-ascorbic acid (Vitamin C, MW = 176.12 g/mol, ACS reagent, purity ≥ 99%) were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Other reagents were of analytical grade from Merck (Darmstadt, Germany) or similar brand.

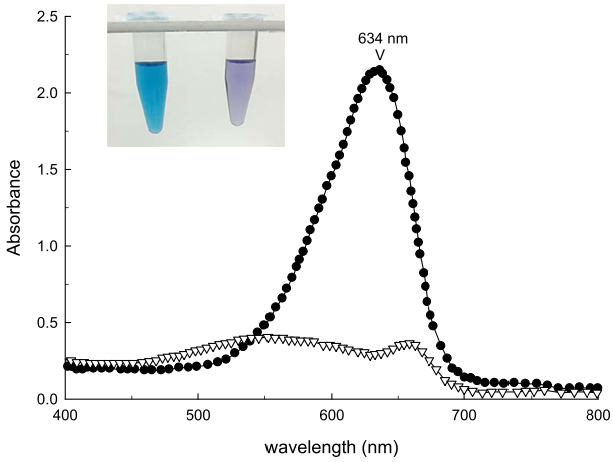

Fig. 3. UV-Vis scanning of the reduced (●, blue tube) and the oxidized (▽, violet vial) forms of BCB. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

BCB detection

BCB detection was performed by UV-VIS spectrophotometry. BCB samples were scanned from 400 to 800 nm to determine the maximum absorbance under our experimental conditions. The maximum BCB wavelength was found at λ_{max} = 634 nm. The BCB absorbance is correlated linearly with the concentration in the range of 0.01–15 μM (Fig. 1S). The BCB solution is blue and becomes violet after oxidation (Fig. 3).

Antioxidant activity of the compounds

The antioxidant activity of molecules was observed by their ability to prevent the dye oxidation in the presence of NaClO (strong oxidant agent). First, a BCB stock solution (1.0 mM) was prepared in water-ethanol (1–3,v/v) solution. Ten microliters were added to a final volume of 1.0 ml of the reaction media (ethanol 75 vol%) containing different concentrations of the samples to be tested. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), resveratrol (RSV), dithiothreitol (DTT), *N*-acetyl-L-cisteine (NAC) and L-glutathione (GSH) were used as control molecules with well-known antioxidant properties. The stocks were freshly prepared in 75 vol% EtOH solution and protected from light exposure until further use. Then, to start the oxidation reaction, 10 μl of 37 mM NaClO was added to each vial, immediately vortexed and incubated in the darkness for 5 min at 25 °C. Finally, the remaining BCB concentration was determined spectrophotometrically at 634 nm (at any time during the first 24 h) and correlated with a proper calibration curve (Fig. 1S). The relative antioxidant activity (AA) was defined as follows:

$$AA(\%) = \frac{(C_m - C_{oxid}) \times 100}{C_m - C_{oxid}} \quad (1)$$

where C_m is the concentrations of BCB containing the molecule to be

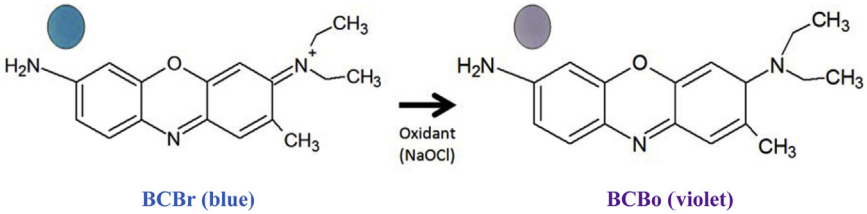

Fig. 2. Scheme of the reduced (BCBr) and oxidized (BCBo) structures of the Brilliant Cresyl Blue (BCB). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Comparative analysis of the weakness/strengths and features of the BCB assay and other antioxidant methods.

	BCB	DPPH	ABTS	References
Sensitivity	1–10 μM	1–10 μM	1–10 μM	Behrendorff et al., 2013 [18] Tan et al., 2016 [27] Gülçin, 2010 [31]
Simplicity	High (one step)	High (one step)	Medium (two or more steps)	Tan et al., 2016 [27] Çelik et al., 2010 [32]. Prior et al., 2005 [7]
Time	5 min	30 min	5–30 min (12 h)[a]	Floegel et al., 2011 [19] Çelik et al., 2010 [32]. Prior et al., 2005 [7]
Cost	Low (9.3 USD/g)[b]	Medium (61.2 USD/g)[b]	Medium (32.5 USD/g)[b]	–
Reagents	Brilliant Cresyl Blue (BCB) -Sodium hypochlorite (NaClO)	2,2-Diphenyl-1-picrylhydrazyl (DPPH)	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) -Potassium persulfate (K2S2O8)	–
Concentration	0.01 mM	0.1 mM	0.1–0.7 mM	Wojtunik et al., 2014 [17] Çelik et al., 2010 [32].
Wavelength	634 nm	517 nm	734 nm	
Solvents	ethanol	methanol	Methanol/others	Wojtunik et al., 2014 [17] Çelik et al., 2010 [32]
Weakness	Low solubility for high lipophilic antioxidants (*i.e.* vitamin E).	Non-physiological radical. DPPH is insoluble in water. Spectra overlapping with some antioxidants (*i.e.* carotenoids).	Non-physiological radical. Time-consuming. ABTS radical generation.	Amorati and Valgimigli, 2015 [33] Prior et al., 2005 [7]
Strengths	Rapid, simple and low cost. Employs a physiological oxidant (NaClO). Highly sensitive	Rapid, simple. Highly sensitive	Wide variety of solvents and wide pH range. Sensitive.	Prior et al., 2005 [7] Moon et al., 2009 [6]

[a] Including generation of ABTS radical after overnight $K_2S_2O_8$ oxidation.
[b] Obtained from Sigma Aldrich Web site (March, 2018). The values reflect the cost of the main reactive: BCB (MW = 385.96), DPPH (MW = 394.32) and ABTS (MW = 548.68).

tested in the presence of the oxidating agent; C_{tot} and C_{oxid} are the concentrations of BCB in the absence of the molecule to be tested without and with the redox agent respectively.

The $\mathbf{AA_{50}}$ is defined as the concentration of the tested molecule providing a 50% protection against BCB oxidation.

Effect of monoterpene concentrations on antioxidant activity

Once the antioxidant properties were established, the relationship between the different monoterpene concentrations and the antioxidant response was evaluated. Concentrations ranging from 6.48×10^{-3} mM to 648 mM were tested for linalool, 1,8-cineole and geraniol.

Assay sensitivities

Limit of detection (LOD) and limit of quantification (LOQ) were used to determine the sensitivity of the spectrophotometric method. LOD and LOQ are defined as the lowest detectable analyte concentration and the lowest analyte concentration determined with appropriate precision and accuracy respectively. The LOD and LOQ were estimated following the equations:

$$LOD = 3 \times \sigma/S \quad (2)$$

$$LOQ = 10 \times \sigma/S \quad (3)$$

where σ is the standard deviation of the response and S is the slope of the curve [11].

Precision

The precision of the method was expressed as relative standard deviation (RSD, %) and relative error (RE%) at three concentrations that correspond to low (around the LOD), mid (two-threefold higher than the LOD) and high (six-ninefold higher than the LOD) levels of the calibration curves. The RE% was calculated as follows:

$$RE\ (\%) = (\text{measured value-theoretical value}) \times 100/\text{theoretical value}\ () \quad (4)$$

Statistic analyses

Three independent experiments were conducted with a minimum of triplicates (N = 3) for each data point. Data for these measurements were analyzed using a Student's *t*-test. Statistically significant values were defined as $p < 0.05$. Data in the graphs represent the mean plus standard deviation.

Results and discussion

Setting antioxidant activity with standards

The BCB was used as tracer shifting from blue to violet when redox agents were added, indicating the presence of oxidized dye species (Fig. 2). The present BCB assay belongs to the category of electron/radical scavenging [6]. The blue color (λ_{max} = 634 nm) of BCB decreased by the addition of an oxidant compound (NaClO), and the protective AA of different molecules was calculated following Eq. (1). Sodium hypochlorite was selected as a strong oxidant able to reduce the time required to determine the AA. In comparison with other oxidants, hypochlorite appears as the best candidate because it shows a strong oxidant activity (just 37 mM is used) and the assay can be read in only 5 min. In addition, NaClO is a low-cost reagent and can be easily purchased from the market.

In other trials, the oxidant activity of hydrogen peroxide (H_2O_2) was tested against the reduced form of BCB (BCBr), but no oxidation was observed, even at a concentration of 979 mM for 24 h (data not shown).

The reaction is fully complete after 5 min and the absorbance can be measured even after the first 24 h since the oxidized products are stable in time. This is an important advantage in comparison with other methods as is described in Table 1 [18].

This method has a limitation when insoluble compounds are tested.

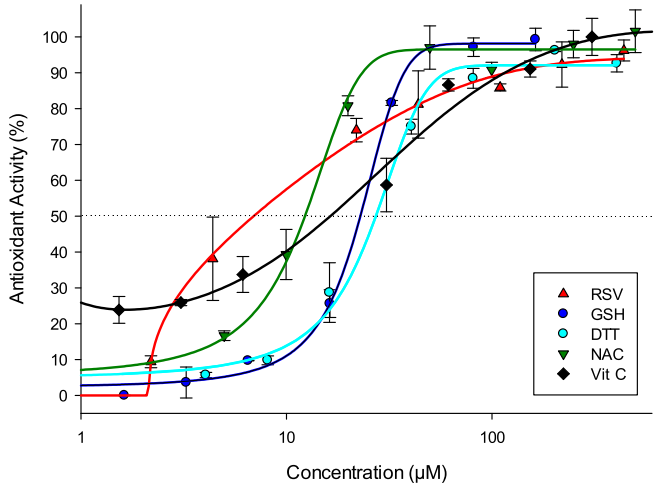

Fig. 4. Antioxidant activity (AA) of diverse antioxidants determined by BCB assay: Resveratrol (RSV), Glutathione (GSH), Dithiothreitol (DTT), *N*-acetyl-L-cysteine (NAC) and Vitamin C (Vit C) at different concentrations.

In the case of vitamin E (Vit E), the protective effect was observed in the range of 110.3 mM–441.1 mM, reaching an AA of 16.0% for the maximum concentration tested. Although Vit E is a well-known *in vivo* antioxidant, in the present BCB assay it showed a reduced protective effect due to its limited solubility in water medium and subsequent precipitation in the reaction vials (Fig. 2S) [24].

To overcome the limitation of AA due to the poor solubility of monoterpenes, a water-ethanol solvent mixture (25–75) was selected as reaction media. It is important to mention that if another solvent is selected to increase the solubility of the components (*i.e.*, acetone), the oxidant environmental conditions will change. In that sense, the concentration of NaClO must be modified in order to observe a protective effect (standards can help to set up the new assay conditions).

Different well-known antioxidants were used as standards to validate the BCB assay (Fig. 4). Resveratrol (RSV), glutathione (GSH), dithiothreitol (DTT) and *N*-acetyl-L-cysteine (NAC) and Vitamin C (Vit C) at different concentrations showed a dose-AA response behavior. Calculations of parameters defining the dose-AA response were established considering the theoretical best-fit curve superimposed on the data points (Table 2). While Vit C exhibited a hyperbolic behavior (R^2 = 0.99), the rest of the antioxidants followed a sigmoidal tendency (R^2 = 0.99 in all cases). Results showed a strong AA of RSV, NAC, Vit C, GSH and DTT in this order, with AA_{50} values of 6.9, 12.3, 16.5, 23.0 and 27.2 μM respectively. In this regard, RSV exhibited an AA at least 2 times higher than NAC and Vit C, and 4 times the AA of GSH and DTT, demonstrating its antioxidant properties in agreement with previous reports [25]. Furthermore, the great ability of RSV to scavenge HOCl was previously described [26]. In concordance with the DPPH method, the results obtained with the BCB assay demonstrated that the AA of standard positive controls are on the order of μM [27].

Table 2
Parameters defining the dose-response behavior in the AA of different standard antioxidants.

Compound	Dose-response	Range (μM)	Equation*	R^2	AA_{50} (μM)
Vit C	hyperbolic	0.0–306.6	y = 14.5*x/(-0.4 + x) +93.9*x/(27.2 + x)-0.005*x	0.99	16.5
RSV	sigmoidal	2.2–438.0	y = 94.2*(1-exp(-(abs (x-9.7/9.0)^0.4)))	0.99	6.9
GSH	sigmoidal	3.2–60.0	y = 98.1/(1 + exp(-(x-22.7)/6.1))	0.99	23.0
DTT	sigmoidal	4.0–80.0	y = 92.1/(1 + exp(-(x-25.7)/9.0))	0.99	27.2
NAC	sigmoidal	5.0–50.0	y = 96.5/(1 + exp(-(x-12.0)/4.3))	0.99	12.3

* y (AA, %) and x (expressed in μM).
Abbreviations: RSV, resveratrol, GSH, glutathione; DTT, dithiothreitol; NAC, *N*-acetyl-L-cysteine.

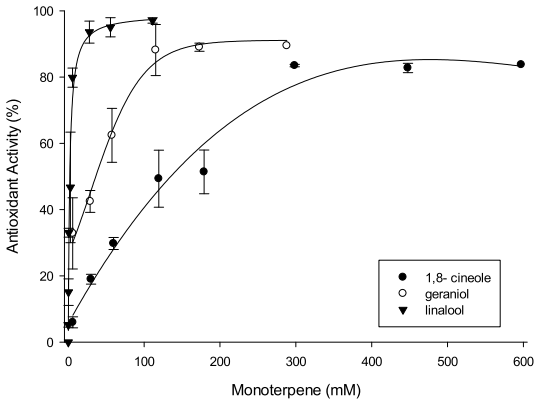

Fig. 5. Antioxidant activity of different monoterpenes determined by BCB assay.

Antioxidant activity of monoterpenes

The monoterpenes tested by the BCB assay showed very interesting AA properties in the milimolar range of concentrations. Particularly, 1,8-cineole exhibited an increased AA as its concentration in the reaction media was raised (Fig. 5). A polynomial behavior was observed in the range of 6.0–450.0 mM with a AA_{50} of 135 mM (Table 3). However, a saturation point was observed at 300 mM, reaching a maximum of 80% BCB protection against hypochlorite oxidation. After that concentration, the solubility of 1,8-cineole began to decrease, similarly as was observed for Vit E, limiting its antioxidant properties. In fact, the BCB solution becomes turbid to the naked eye at 600 mM 1,8-cineole, indicating a poor solubility in aqueous/ethanol media.

On the other hand, the AA activity of geraniol was also evaluated by the BCB assay (Fig. 5). Geraniol showed an AA increasing from 5.8 mM to 175.0 mM with a 90% of BCB protection, describing a perfect sigmoidal behavior (R^2 = 0.99) as is observed in Table 3. The monoterpene showed an $\mathbf{AA_{50}}$ of 36.2 mM, demonstrating an AA that is in the milimolar range and 10,000 to 4000 lower than that of standard antioxidants (Table 2). Similarly as was previously mentioned for Vit E and 1,8-cineole, the AA was limited by geraniol solubility and reached a maximum of 90% protection. Considering that both monoterpenes are soluble at 22.7 mM and 4.5 mM in water at 25 °C (1,8-cineole and geraniol respectively) it was expected that geraniol would first exhibit a saturation point in the AA. In concordance with that observation, the phenomenon was evidenced at 175 mM for geraniol and at 300.0 mM for 1,8-ineole. This fact reflects that the AA detected by the present method is intrinsically correlated with the solubility of the compound in the reaction media.

Finally, the AA of linalool was tested considering previous works reporting the strong antioxidant properties of this compound [28]. In Fig. 5, a strong AA of linalool in the presence of hypochlorite is observed. The AA_{50} value was around 2.3 mM, which represented an AA of 59- and 16-fold higher than that of 1,8-cineole and geraniol respectively. A hyperbolic dose-AA response was observed for linalool (R^2 = 0.98). In this case, the saturation point was almost reached at 111.2 mM, showing an AA strong enough to protect 100% of the dye against oxidation. Although the AA observed for linalool by the BCB assay was from 85 to 333 times lower than that of standard

Table 3
Parameters defining the dose-response behavior in the AA of monoterpenes.

Monoterpenes	Dose-response	Range (mM)	Equation*	R^2	AA_{50} (mM)
1,8-Cineole	linear	6.0–120.0	y = 0.372x + 6.03	0.99	–
	polynomial	6.0–450.0	$y = -0.0004x^2 + 0.337x + 9.750$	0.99	135.0
Geraniol	linear	6.0–115.0	y = 0.515x + 29.74	0.99	–
	sigmoidal	6.0–175.0	y = 91.1/(1 + exp(-(x-29.6)/33.8)	0.99	36.2
Linalool	linear	0.0–1.2	y = 57.31x + 0.57	0.99	–
	hyperbolic	0.1–111.0	y = 3.5 + 95.6x/(2.1 + x)	0.98	2.3

* y (AA, %) and x (expressed in mM).

antioxidants, the values are strong enough to justify its active biological properties in cancer therapy and antimicrobial treatments [29].

The low AA of 1,8-cineole in comparison with the other monoterpenes (4 and 59 times lower than that of geraniol and linalool respectively) is consistent with the lack of double bonds in its molecule and is in agreement with other reports describing the AA of monoterpenes with similar structure [17].

In previous works, the AA of different monoterpenes was evaluated by DPPH and ABTS, and similar results were found in comparison with the BCB assay. Tan et al. (2016) found that c-terpinene, citral and terpinolene exhibited a DPPH radical scavenging activity of 83.0%, 77.9% and 75.3% respectively at a concentration of 100 mM. On the other hand, (R)-(+)-limonene, (R)-(−)-linalool, (S)-(−)-b-citronellol, nerol, geraniol, (S)-(−)-perillyl alcohol and α-terpineol showed a weak radical scavenging effect in the range of 5.3%–18.4%. As a control standard antioxidant, ascorbic acid showed a scavenged DPPH activity of 77.6% at 25 μM, which was significantly more potent than that of monoterpenes. In regard to the ABTS method, different monoterpenes also showed antioxidant activity in the range of 10.4%–34.9% at 100 mM. However, no significant ABTS radical scavenging activity was found for other monoterpenes such as geraniol, l-menthol, nerol, (R)-(−)-linalool and (S)-(−)-b-citronellol [27].

In concordance with DPPH, our current findings with the BCB assay demonstrated that the AA of some monoterpenes fell within the milimolar range, while the AA of standard positive controls was on the order of μM (Tables 2 and 3). As a difference with ABTS, our work demonstrated that geraniol and linalool exhibited AA, which was on the order of 36.2 and 2.3 mM (AA_{50}) respectively.

In particular, from a biological point of view, the BCB assay presents some advantages in comparison with other antioxidant methods for monoterpenes. The present method adopts a better physiological approach (in comparison with DPPH and ABTS) since the AA was determined using an oxidant agent commonly generated in physiological environments (both the DPPH and ABTS assays use nonphysiological radicals as substrate). In fact, hypochlorite is the major strong ROS produced by activated neutrophils and monocytes as host defense mechanism against microorganisms. However, in some conditions it could cause tissue damage and enhance the progression of diseases like atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, chronic inflammation and different types of cancer [19,30].

Quantification of monoterpenes

Among the different methods for monoterpene detection, the UV-Vis is one of the most simple [29]. In this section, the potential utility of the new BCB assay for monoterpene quantitation at mM concentrations is demonstrated in Fig. 6.

In the case of linalool, a linear response was observed in the range of 0.0–1.2 mM (R^2 = 0.99). Low detection limits of linalool were established (LOD = 0.06 mM; LOQ = 0.19 mM), mainly enhanced by its high antioxidant activity. In addition, a good precision of the method was observed at the highest portion of the analytical curve with relative errors lower than 5% (Table 1S).

As is shown in Table 3, there is a linear relationship between AA and geraniol concentration in the range of 5.8–115.0 mM (R^2 = 0.99). The LOD and LOQ for the BCB-geraniol curve were 22.0 and 66.8 mM, and low relative standard deviations were found along the linear range of concentrations tested, suggesting good analytical precision (Table 1S).

Furthermore, a linear relationship between 1,8-cineole concentration and AA (%) was also observed in the range of 6.0–120.0 mM (R^2 = 0.99). Considering that no spectroscopic methods (UV-Vis) for 1,8-cineole are found in the literature, because the molecule does not exhibit fluorescence or UV absorption properties, the present method could be useful to estimate the 1,8-cineole concentration in pure samples. The LOD and LOQ were established at 16.5 mM and 49.9 mM respectively. The method showed RSD (%) and RE (%) lower than 10%, which indicates very good precision at the lowest and mid portion of the analytical curve (Table 1S).

Finally, the standard calibrations curves of absorbance (at the maximum wavelength of each monoterpene) and the linear fit curves of AA versus monterpene concentration were compared. While the conventional UV-Vis method could detect linalool at the maximum wavelenght of 233 nm with a LOD of 3.35 mM, the BCB assay allowed monoterpene quantification with a LOD of 0.06 mM. That difference represents a sensitivity of the BCB assay 56 times higher than that of the conventional UV-Vis. Regarding geraniol detection by both methods, the UV-Vis exhibited a higher sensitivity (LOD 100 times higher) than the BCB assay, but the latter covers a higher range of concentrations. More interesting are the results for 1,8-cineole, which cannot be detected by absorbance since the molecule does not exhibit peaks in the UV-Vis region.

Conclusions

In the present work a simple, low-cost and rapid method was developed for the determination and comparison of the antioxidant activity of different monoterpenes. The ability of these molecules to prevent the oxidation of a dye (BCB) in the presence of a strong oxidant such as sodium hypochlorite was tested, and the results suggested a protective effect of the monoterpenes.

A shift of the typical blue color of the dye (λ_{max} = 634 nm) to violet was observed in the presence of the oxidant in only 5 min, and the final delay in that change was used to track the AA. Different well-known antioxidants (Vit C,Vit E, RSV, NAC, DTT and GSH) were used as controls to determine a correlation between concentrations and antioxidant response.

Linalool, geraniol and 1,8-cineole were tested and showed very different antioxidant properties in a dose-dependent manner. The concentrations that provide a 50% protection against oxidation (AA_{50}) were 2.3, 36.2 and 135.0 mM respectively, indicating a strong AA of linalool.

This method provides a simple, fast (only 5 min), reliable and sensitive spectrophotometric technique with minimal equipment requirements to determine the *in vitro* antioxidant activity of soluble molecules in the reaction media. In addition, the technique is a very useful tool to quantify the amount of monoterpenes in a defined range of milimolar concentrations considering their AA.

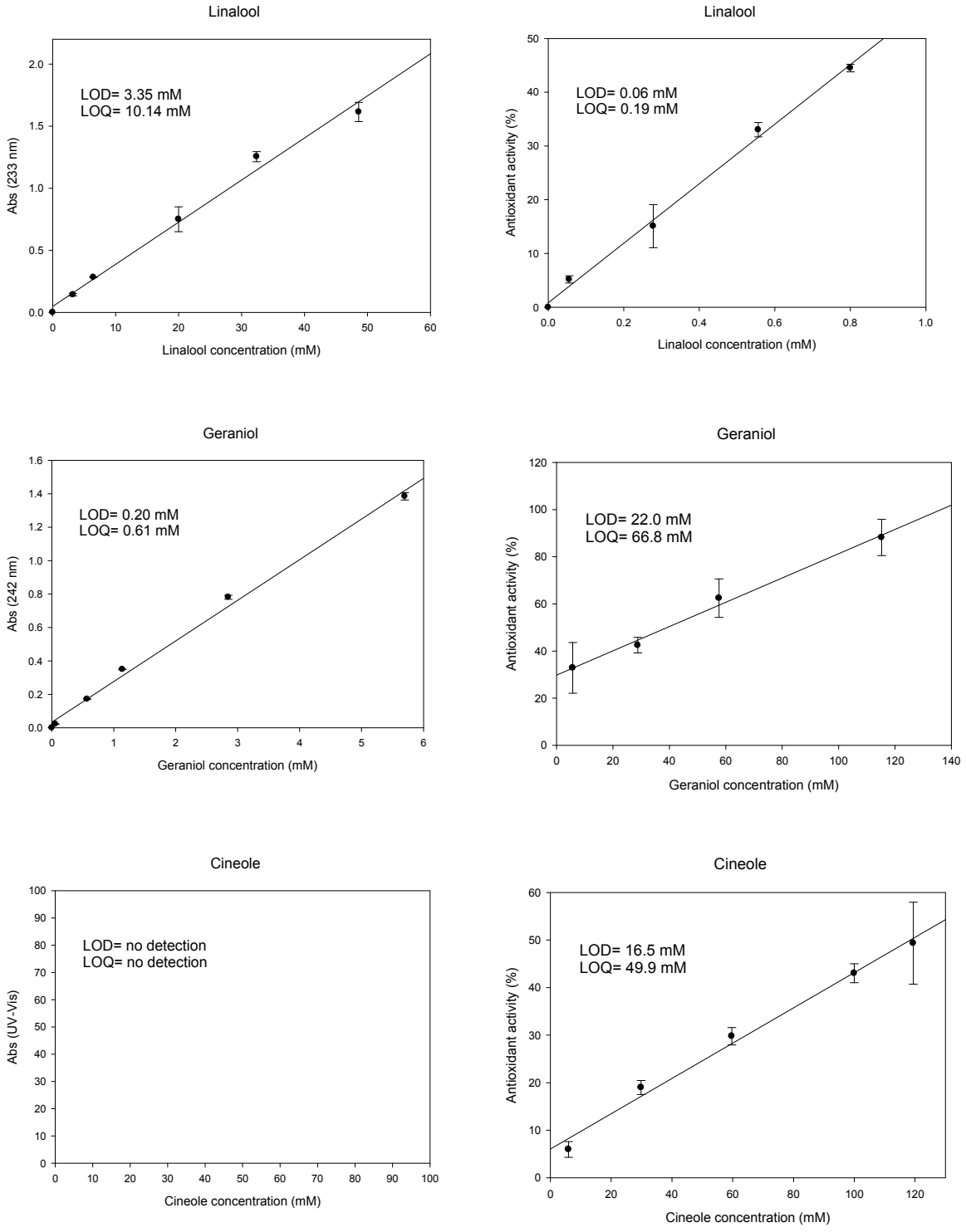

Fig. 6. Comparison of methods for monoterpenes determination: UV-Vis spectroscopy (left) and the BCB assay (right). The limits of detection (LOD) and limits of quantification (LOQ) of each method are indicated inside the graphic.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2018.06.007.

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