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Contribution of \textit{trans}-aconitic acid to DPPH$^*$ scavenging ability in different media

William Piang-Siong$^a$, Pascale de Caro$^{b,c}$, Arnaud Marvilliers$^a$, Xavier Chasseray$^a$, Bertrand Payet$^a$, Alain Shum Cheong Sing$^{a,*}$, Bertrand Illien$^a$

$^a$ Université de la Réunion, Faculté des Sciences et Technologies, LCSNSA (Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments), 15 avenue René Cassin, CS 92003 – RE-97744 Saint Denis Cedex 9, La Réunion, France
$^b$ Université de Toulouse, INP, LCA (Laboratoire de Chimie Agro-Industrielle), ENSIACET, 4 allée Emile Monso, F-31030 Toulouse, France
$^c$ INRA, UMR 1010 CAI, F-31030 Toulouse, France

A B S T R A C T

The antioxidant properties of \textit{trans}-aconitic acid (TAA) alone or in the presence of usual antioxidants were assessed by DPPH$^*$ assay. The IC$_{50}$ value equal to 70 mM was very high compared to usual antioxidants (vitamin C and trolox). A joint experimental/theoretical study suggested that hydrogen atom abstraction in TAA by DPPH$^*$ was located on –CH$_2$– methylene bridge because the corresponding radical was more stabilized than COO$^\cdot$ and C=C$^\cdot$ radicals. In combination with antioxidants (vitamin C, gallic acid, caffeic acid, trolox), synergy or additivity effects were noticed. The magnitude of the synergistic effect varied between 1.06 and 1.24 depending on the type and concentration of antioxidant for a concentration of TAA equal to 22.3 mM. Especially, the addition of TAA at a concentration below 32 mM to a solution containing 20 $\mu$M of vitamin C had a synergy effect. Beyond this concentration, TAA showed an additive effect.

1. Introduction

Antioxidants are listed as additives in food (Perrin & Meyer, 2002; Shahidi, 2000), cosmetic (Alander, Andersson, & Lindström, 2006) and pharmaceutical (Celestino et al., 2012) products for the properties they generate in biological and organic media. They are either of synthetic origin (BHT, BHA, propyl gallate) (Freitas & Fatibello-Filho, 2010), or of natural origin (ascorbic acid, vitamin E, carotenoids, polyphenols) (Bruun-Jensen, Skovgaard, Madsen, Skibsted, & Bertelsen, 1996; Martí, Pérez-Vicente, & García-Viguera, 2002). These natural antioxidants present in the plant extracts are increasingly sought to replace the synthetic compounds (Gramza & Korczak, 2005; Wagner, Wotruba, & Elmadfa, 2001). The advantage of natural extracts is to associate one or more antioxidants with other metabolites that often have promoting effects on the antioxidant activity. Thus, the role of certain organic acids, in particular polyacids, was recently highlighted. Methanolic extracts of chamomile with a high content of phenolic acids, flavonoids and organic acids showed a strong antioxidant potential (Guimarães et al., 2013). Phenolic compounds and organic acids may contribute to the biological activity of Brazilian tropical fruit. 

* Corresponding author.
E-mail address: alain.shum@univ-reunion.fr (A. Shum Cheong Sing).
The antioxidant activity of turnip extracts was correlated with the total amount of phenolic compounds and organic acids (Fernandes et al., 2007). Specifically, citric acid added to a rosemary extract containing rosmarinic acid (antioxidant) showed a synergistic effect to prevent hydroperoxide formation (Hraš, Hadolin, Knez, & Bauman, 2000). Similarly, the simultaneous presence of oxalic acid, citric acid and malic acid enhanced the antioxidant activity of an extract of the roots of Madagascar periwinkle (Pereira et al., 2010). IC₅₀ value of the extracts of quince (such as pulp, peel and jam) can be correlated with the concentration of vitamin C and citric acid (Silva et al., 2004). A model system was developed to test the influence of a few acids (acetic, malic and citric acids) on ascorbic acid by the DPPH test (Scalzo, 2008). Other polyacids (itaconic acid, cis-and trans-aconitic acid or fumaric acid) are often present in the acid fraction along with acetic, malic or citric acids in the extracts or in certain food or cosmetics preparations (Blass, Pratt, & Cosentino, 2013; Silva, Azevedo, Pereira, Valentão, & Andrade, 2013). However, their contribution to biological activity remains unclear.

Most of these acids can be obtained on an industrial scale by biotechnological processes or by extraction of industrial by-products (Cao, Du, Gong, & Tsao, 1996; Li & Punt, 2013). Knowledge of their contributions to the antioxidant activity would bring new development prospects for these products. In this perspective, aconitic acid, the major acid representing approximately 60% of organic acids in sugar cane molasses, was studied (Célestine-Myrtil & Parfait, 1988). Aconitic acid can also be obtained by dehydration of citric acid (Bruce, 1937; Cranston, 1951, 1955). It is naturally present in sugar cane and can be isolated from by-products of the sugar industry (molasses, vinasse) (Malmary, Albet, Putranto, Hanine, & Molinier, 2000; Montoya, Londono, Cortes, & Izquierdo, 2014; Petit et al., 2015; Pislor, Pontalier, & Albet, 2009). The amount available in the molasses is between 7.1 g/kg molasses in Senegal to 23.4 g/kg in Louisiana (Grondin, Albet, 2009). A blank prepared with 20 μl methanol in 280 μl of DPPH solution (ranging from 8.62·10⁻⁴ M to 0.1 M) were prepared. 20 μl of the solution to be tested were introduced in each of the 96 wells of the microplate. Then, 280 μl of DPPH at 0.004% (4 mg/100 ml) in methanol were added. Microplate was incubated for 1 h at 30 °C and absorbance was measured at 515 nm. A conical fermenter with 20 μl methanol in 280 μl of DPPH was also taken through the same procedure to determine its antioxidant capacity.

The radical-scavenging activity of the samples expressed as an inhibition percentage was calculated according to the absorbance values;

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

where \(A_{\text{blank}}\) is the absorbance of the blank and \(A_{\text{sample}}\) is the absorbance of the product, at 515 nm, after one hour. Inhibition percentage increases with antioxidative activity.

The concentration for which 50% inhibition is obtained, is called inhibition concentration (IC₅₀). It was calculated from the graph obtained by plotting “inhibition percentages” versus “sample concentrations”.

For the kinetics study of a sample (mixture of antioxidant and TAA), measurements of absorbance at 515 nm were performed, immediately after addition of DPPH, at five minutes intervals for one hour.

### 2.3. Computational study

In order to get an insight into the activity of TAA on DPPH scavenging, radicals of TAA with different positions of hydrogen atom abstraction (Fig. 1) were theoretically studied. Geometries were optimized at the B3LYP/6-31+G(d,p) level using the Gaussian 09 program (Frisch et al., 2013) and tight convergence criteria. An ultrafine grid was used to ensure rotational invariance of the results. All stationary points were confirmed as true minima via vibrational frequency calculations in the harmonic approximation. The popular B3LYP Hybrid density functional (Becke, 1993; Stephens, Devlin, Chabalowski, & Frisch, 1994) is widely used to study organic compound geometry (Sousa, Fernandes, & Ramos, 2007). 6-31+G(d,p) Pople style basis set is large enough to describe the structure of organic molecules (even for hydrogen-bonded complexes) at this B3LYP level (Koné, Illien, Graton, & Laurence, 2005).
Then G4(MP2)-6X composite procedure was applied to improve relative energies of the most stable radical conformers. For 28 hydrogen-abstraction reactions, this procedure achieves 1.7 kJ mol\(^{-1}\) \(\Delta H\) (0 K) mean absolute deviation to W1 reference values (Chan, Deng, & Radom, 2011).

2.4. Statistical analysis

XLSTAT-Pro Version 2007.4 (Addinsoft, France) was used to calculate Student-Fisher and Friedman tests.

3. Results and discussion

3.1. Experimental and theoretical study of the radical scavenging activity of aconitic acid

The DPPH\(^{•}\) test was used to monitor the inhibition percentage versus aconitic acid concentrations. It was then possible to deduce the value of IC\(_{50}\), found to be equal to 70 mM corresponding to the effective TAA concentration for 50% inhibition (Supplementary data – Fig. S1). Nevertheless, compared to IC\(_{50}\) values of conventional antioxidants, such as vitamin C (24.4 \(\mu\)M) and trolox (16.1 \(\mu\)M) (Payet, 2005), the IC\(_{50}\) of the aconitic acid is rather high. We can deduce from these results that aconitic acid has weak radical scavenging properties and cannot therefore be considered as an effective antioxidant.

In order to investigate if acid functions of TAA (pK\(_{a1}\) = 2.80; pK\(_{a2}\) = 4.46; pK\(_{a3}\) = 6.30) are involved in the scavenging process, DPPH\(^{•}\) tests were also applied to acetic (pK\(_{a1}\) = 4.75) and citric acids (pK\(_{a1}\) = 3.13; pK\(_{a2}\) = 4.76; pK\(_{a3}\) = 6.40). Inhibition percentages at 0.1 M concentration were found (several different starting points had led to the same geometrical parameters). Sketches, energies and geometrical parameters for the most stable COO\(^/-\)/C\(_5\) and C\(_2\) radical species were gathered in Table 1. The results for the other 18 radical species can be found in Supplementary material (Table S2). Five HC\(_3\) conformers were optimized. Their relative energies (\(\Delta E = 0–14 \text{ kJ/mol}\) were lower than those of the twelve COO\(^/-\)/C\(_5\) species (\(\Delta E = 89–100\) ) and of the four C\(_2\) conformers (107–118). In HC\(_3\) radical, C\(_1\), C\(_2\), C\(_3\), C\(_5\) and C\(_6\) atoms were almost in the same plane; C\(_2\)C\(_3\) and C\(_5\)C\(_6\) bond lengths were nearly equal, respectively 139.6–139.9 and 139.2–139.6 pm. Thus the higher stability of HC\(_3\) radical came from a better delocalization of \(\pi\) electrons over carbon skeleton than in COO\(^/-\)/C\(_5\) and C\(_2\) radicals. Accurate relative G4(MP2)-6X \(\Delta H\) (0 K) values for molecules in Table 1 showed the same trend as relative B3LYP energies. In a nutshell, hydrogen atom abstraction in TAA by DPPH\(^{•}\) was located on –CH\(_2\)– methylene bridge because the corresponding radical was more stabilized than COO\(^/-\)/C\(_5\) and C\(_2\) radicals. Therefore the following reaction mechanism of DPPH\(^{•}\) with TAA can be suggested (Fig. 2).

3.2. Kinetic study of aconitic acid solution mixed with conventional antioxidants

Four conventional antioxidants were selected to determine the behaviour of aconitic acid when mixed with each of them. The concentration of aconitic solution was 22.3 mM (Scalzo, 2008). The results of this kinetic study were reported in Fig. 3 (part a, b, c).

First, kinetics of the reaction between each of the four standard antioxidants and DPPH\(^{•}\) were monitored by measuring inhibition percentages (part a). We note that the inhibition percentages are nearly constant over time. The percentage of inhibition has levelled off at 36.1 ± 1.3, 44.9 ± 0.8, 47.7 ± 0.7, 48.0 ± 1.7 for caffeic acid, gallic acid, vitamin C and trolox respectively.

In part b, kinetic profiles from mixtures between a standard antioxidant and aconitic acid are presented. In these cases, the time required to reach the maximum value for inhibition percentage depends on the nature of the antioxidant: the first part of Fig. 3b indicates that the reaction kinetics between the antioxidant and radical DPPH\(^{•}\) is slowed for several tens of minutes. However, the second part shows a levelling off of the percentage of inhibition, indicating the end of the reaction between the antioxidant and radical DPPH\(^{•}\). Slower kinetics of reaction between an antioxidant (phenols, curcumin or vitamin E) and DPPH\(^{•}\) radical in the presence of acetic acid was reported by several authors.

![Fig. 2. Possible reaction mechanism of TAA with DPPH.](image-url)
As the attack of the DPPH• on the antioxidant acid is limited, the mechanism of proton transfer preferentially takes place. This later occurs at a slow rate, which explains the kinetic profile observed in the presence of aconitic acid. The results are then consistent with the SPLET mechanism.

The threshold values obtained (51.7 ± 0.6, 57.1 ± 0.6, 64.8 ± 0.7, 66.3 ± 0.6 for caffeic acid, gallic acid, vitamin C and trolox respectively) are higher than the inhibition percentages observed for the standard antioxidants (part a). It means that mixtures with aconitic acid lead to higher inhibition percentages after a latency period (20–50 min depending on the antioxidant).

3.3. Comparison of threshold values of inhibition percentage of mixtures

Table 2 contains the maximum values of inhibition percentages obtained for mixtures containing an organic acid (acetic acid, citric acid and aconitic acid) and usual antioxidants (vitamin C, caffeic acid, gallic acid and trolox).

The samples with methanol, without any addition of acid, represent the blank samples. A Friedman test was performed (threshold risk α = 5%) to observe the differences in activity caused by the addition of an acid, compared to the acid free sample. The addition of acetic acid or citric acid does not generate significant differences compared to the blank except for the mixture, trolox + citric acid, which has a relatively small difference (4.9%). Then, for a given antioxidant, firstly, the inhibition percentages increase with the antioxidant concentrations, secondly, the inhibition percentages obtained with the addition of acetic acid and citric acid are quite similar. Lo Scalzo (Scalzo, 2008) found the same trends for vitamin C in combination with acetic and citric acid in ethanol for the same concentrations, but inhibition percentages were different. It is well known that DPPH test is sensitive to solvent and an antioxidant (ROH) according to SPLET mechanism and hydrogen transfer.

Fig. 3. Kinetic studies of aconitic acid solution mixed with conventional antioxidants (15 μM caffeic acid, 5 μM gallic acid, 22.5 μM vitamin C and trolox). (a) Inhibition percentages of standard antioxidants versus time. (b) Inhibition percentages of different antioxidants in solution, in association with an organic acid at 0.067 N in methanolic solution. (c) Reaction between DPPH and an antioxidant (ROH) according to SPLET mechanism and hydrogen transfer.

(Scalzo, 2008). Based on the reaction mechanisms involving the DPPH, this result can be explained with the SPLET mechanisms coupled with the hydrogen transfer (part c). The SPLET mechanism is initiated by the formation of an alkoxide or a phenoxide, and has a high constant reaction rate. Given the pKa of aconitic acid (pKa = 2.80), pH of the solution is low and ROH form predominates.

Table 2

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration (μM)</th>
<th>Blank</th>
<th>Acetic acid (67 mM)</th>
<th>Citric acid (22.3 mM)</th>
<th>Relative gain in antioxidative activity with TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>15</td>
<td>29.1 ± 1.7</td>
<td>32.6 ± 0.5</td>
<td>33.9 ± 1.1</td>
<td>46.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>48.0 ± 1.7</td>
<td>52.2 ± 0.7</td>
<td>51.6 ± 1.3</td>
<td>64.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>91.7 ± 0.2</td>
<td>92.0 ± 0.1</td>
<td>90.9 ± 1.3</td>
<td>91.8 ± 0.2</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>5</td>
<td>17.9 ± 1.8</td>
<td>16.9 ± 0.8</td>
<td>19.4 ± 0.6</td>
<td>37.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>36.1 ± 1.3</td>
<td>34.9 ± 0.4</td>
<td>38.7 ± 1.4</td>
<td>51.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>56.6 ± 1.6</td>
<td>55.8 ± 1.4</td>
<td>52.4 ± 1.6</td>
<td>68.9 ± 1.0</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2</td>
<td>10.2 ± 1.1</td>
<td>12.0 ± 1.1</td>
<td>14.3 ± 0.5</td>
<td>30.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>44.9 ± 0.8</td>
<td>46.2 ± 0.6</td>
<td>46.2 ± 1.2</td>
<td>57.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>61.7 ± 1.1</td>
<td>61.5 ± 1.1</td>
<td>56.2 ± 1.4</td>
<td>67.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>28.1 ± 1.8</td>
<td>30.4 ± 0.7</td>
<td>33.0 ± 1.3</td>
<td>46.5 ± 0.5</td>
</tr>
<tr>
<td>Trolox</td>
<td>22.5</td>
<td>47.7 ± 0.7</td>
<td>48.5 ± 0.6</td>
<td>53.7 ± 0.5</td>
<td>66.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>91.6 ± 0.3</td>
<td>91.7 ± 0.1</td>
<td>90.8 ± 0.6</td>
<td>91.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Significant difference according to Friedman test (P = 0.05 and n = 6).
The synergistic effect (SE) of a mixture is defined by the ratio of the experimental value of the inhibition percentage of the mixture (% I mixture) and the theoretical value (% I theoretical) (Liu, Shi, Ibarra, Rose, & Narishetty, 2012; Roy, Berardi, Chan, & Lee, 2013; Roy, Noguchi, Tsuchihashi, & Gotoh, 1995) or by its activation of anti-oxidant properties of natural extracts should take into agreement with a HAT mechanism.

Synergetic effect has been studied particularly for vitamin C at a concentration of 20 μM. For this antioxidant commonly used in food industry, the synergistic effect of aconic acid is active below a concentration of 32 mM. Beyond this value, the additive effect replaces the synergetic effect. This result shows that the measurement of anti-oxidant properties of natural extracts should take into account both the contents of antioxidant molecules and organic acids.

### Acknowledgments

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### Table 3

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration (μM)</th>
<th>%I mixture</th>
<th>%I theoretical</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>15</td>
<td>46.1 ± 1.6</td>
<td>40.6 ± 1.6</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>64.8 ± 0.7</td>
<td>56.4 ± 1.3</td>
<td>1.15</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>5</td>
<td>37.8 ± 1.0</td>
<td>31.1 ± 2.0</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>51.7 ± 0.6</td>
<td>46.4 ± 1.4</td>
<td>1.11</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2</td>
<td>30.6 ± 0.9</td>
<td>24.7 ± 0.8</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>51.7 ± 0.6</td>
<td>53.8 ± 0.9</td>
<td>1.06</td>
</tr>
<tr>
<td>Trolox</td>
<td>15</td>
<td>46.5 ± 0.5</td>
<td>39.7 ± 0.6</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>66.3 ± 0.6</td>
<td>56.1 ± 0.7</td>
<td>1.17</td>
</tr>
</tbody>
</table>

* Significant difference according to Student-Fischer test (P < 0.05 for n = 6) to compare mixture percentages and theoretical percentages.

### 4. Conclusion

Already known for its acidifying properties, trans-aconitic acid had never been studied for its antioxidant properties. The method of DPPH test helped to highlight a too low radical scavenging activity to be exploited; an IC50 value of 70.4 μM was found for TAA, less than that of vitamin C (IC50 = 24.4 μM) or that of trolox (IC50 = 16.1 μM), an analogue of vitamin E. The theoretical study showed that carboxylic acid moiety is probably not involved in the reaction mechanism with DPPH. In fact, hydrogen atom abstraction in TAA by DPPH was located on –CH2– methylene bridge because the corresponding radical was more stabilized than COO– and C=C radicals.

A synergetic effect resulted from the combination of trans-aconic acid and an antioxidant, such as vitamin C, gallic acid, caffeic acid, while the combination with citric acid and acetic acid showed no effect under the same conditions. Note that the antioxidant concentration should not be too high to allow synergetic effect. This property has the advantage of enhancing the effectiveness of the tested conventional antioxidants, which could be used at lower concentrations. Kinetics of reactions of DPPH with the antioxidants, in the presence of aconic acid, have shown a latency period before reaching a stable inhibition percentage, which is in agreement with a HAT mechanism.

### 3.5. Determination of an effective maximum concentration of aconitic acid

Vitamin C was chosen as a standard antioxidant to study the influence of the concentration of aconitic acid on the synergistic effect of the mixture. The concentration of vitamin C was set at 20 μM and two ranges of concentrations in aconitic acid were selected according to potential food additive applications or antiparasitic formulations: between 10 and 800 μM (Chubb, de Rose, & Narishetty, 2012; Roy, Berardi, Chan, & Lee, 2013; Roy, Letourneau, Culver, & Behrens, 2012; Takase et al., 2007) and between 6 and 60 mM (Moriwaki, Shimizu, Nishide, & Koike, 2009) (Supplementary data – Fig. S3). In the food range, the SE remains constant (between 1.19 and 1.23) despite the increase in aconitic acid. But, a slight decrease of the SE is observed on the second range.

To determine the maximum effective concentration in aconitic acid, a gain in antioxidant activity due to the contribution of aconitic acid is calculated using the following formula:

\[
\text{gain} = \frac{\% I_{\text{mixture}} - \% I_{\text{vitamin C}}}{\% I_{\text{theoretical}}}
\]