



## ***N*-Acetyl-cysteine Increases Chemical Stability of Hydroquinone in Pharmaceutical Formulations: a Theoretical and Experimental Approach**

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In this study, the chemistry stability of hydroquinone (HQ) was evaluated according to its effects in redox properties and compared to kojic acid (KA). The HQ oxidation was more inhibited by *N*-acetylcysteine (NAC) than ascorbic acid (AA). These results were elucidated using theoretical methods at the DFT/B3LYP level of theory. All electronic parameters were related between antioxidant performance and highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), HOMO-LUMO value gap (GAP), ionization potential (IP), and phenol or enol bond dissociation energy (BDE<sub>OH</sub>) values. However, the interactions between HQ and NAC cannot be related by changing of these electronic parameters. Therefore the high calculated values for electron transfer can be associated to NAC due to polarizability or chelation properties of sulfur moiety.

**Keywords:** hydroquinone, *N*-acetylcysteine, stability, antioxidant, molecular modeling

### **Introduction**

Chemical stability of pharmaceutical molecules is a great problem that can affect its safety and efficacy as drug product. Besides the necessary metabolic evaluation related to ADMET (absorption, distribution, metabolism, excretion and toxicity) stability properties, the drug or candidate should also have excellent stability in hydrolysis, oxidation, photolytic and thermal conditions.<sup>1</sup> Nonetheless, a previous study of chemical stability of molecule helps mainly in selecting formulations processes.

Tyrosinase is a metalloenzyme involved in the formation of pigments such as melanin and other polyphenolic compounds.<sup>2</sup> This enzyme uses the molecular oxygen to catalyze the oxidation of monophenols to its corresponding *o*-diphenols by using monophenolase or cresolase activities. Then, the enzyme catalyze the subsequent oxidation to their respective *o*-quinones by diphenolase or catecholase activities.<sup>3</sup> The tyrosinase is responsible in mammals for skin pigmentation<sup>4,5</sup> and it is linked to Parkinson disease and other neurodegenerative diseases.<sup>6,7</sup> In addition, skin pigmentation disorders

consist of an overproduction or irregular distribution of melanin, resulting in skin spots<sup>8</sup> due to numerous factors,<sup>9</sup> including the use of certain drugs.<sup>10</sup>

Topical agents, chemical peels, cryotherapy and laser therapy are the main options for the treatment of skin hyperpigmentation.<sup>11,12</sup> However, the most popular treatment utilized for depigmentation is the topical cosmetics containing hydroquinone (HQ), the main therapeutic options in their formulation substances, as well as arbutin, azelaic acid, and kojic acid,<sup>13,14</sup> other agents acting through different mechanisms.<sup>15,16</sup>

The effectiveness of HQ is related directly to the concentration of the preparation in the vehicle used to the chemical stability of the final product. Its concentration varies from 2% up to 10% in formulation. Nevertheless, the chemical stability of HQ formulations is important because it is easily oxidized and loses its potency. Therefore, antioxidants such as 0.1% sodium bisulfate and 0.1% ascorbic acid should be used to preserve the stability of the formulation.<sup>16,17-19</sup> The possible reactions of the HQ degradations are mediated by radical oxygen and its proposed oxidation mechanisms to give semiquinone and quinone forms. These intermediates are responsible for the hydroquinone polymerization reactions.<sup>16,19</sup>

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In recent years, studies of tyrosinase inhibition activity have had great interest and many compounds have been tested due to their industrial impact. Furthermore, the extensive economic value of the inhibitors and different inhibitor compounds derived especially from natural products or synthetic compounds, have increased this interest.<sup>11,14,15</sup> Since hydroquinone (HQ), kojic acid (KA) and ascorbic acid (AA) are simple structures, these molecules are an attractive target for chemical stability studies due to their structure-property and design of better formulations. Therefore, in this work the increase of chemical stability of HQ by thiol antioxidant compound is displayed using experimental and theoretical methods.

## Experimental

### Chemical reactants

The following reactants and chemicals were used: kojic acid, sodium bicarbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ( $\pm$ )-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich, while hydroquinone and ascorbic acid were obtained from Merck Co. All reagents used in this work have analytical grade ( $\geq 98$ -99% TLC/HPLC (thin layer chromatography/high performance liquid chromatography)).

### Chemical stability

All compounds were formulated in concentration of 5% on cream and/or gel, i.e., (i) hydroquinone, (ii) kojic acid, (iii) ascorbic acid, and (iv) hydroquinone + N-acetylcysteine (NAC) association (0.05%). All formulations were stable except the hydroquinone cream and gel. The chemical stability study was executed at temperature of 29-35 °C. The same conditions were used in water or water:ethanol solutions. However, no alteration was observed. Thus, the oxidation reaction was accelerated by alkalization using sodium bicarbonate 0.05% solution (water:ethanol 1:1).<sup>1,20</sup> The absorbance variation was observed in 400 nm. All experiments were performed at least three times.

### Antioxidant evaluation

A methanolic solution of the DPPH radical (0.04%) was prepared daily and protected from light. Absorbance at 517 nm was recorded to check the stability of the radical throughout the time of analysis. The effect of HQ and related derivatives on the DPPH absorbance was estimated

by using the previous procedure.<sup>21,22</sup> The trolox (TLX) was used as positive control.

### Statistical analysis

All experiments were performed at least three times. The kinetic reactions of HQ and related derivatives were related with antioxidant and chemical stability. The polymerization level or DPPH inhibition were related to control TLX and NAC.

### Theoretical study

The aim of this research is the elucidation of the hypothetical mechanism of hydroquinone oxidation by using theoretical methods. Our experience on theoretical methods for chemistry reactivity studies<sup>21-23</sup> has encouraged us to examine this process in this context.

Here, the geometry optimizations of the hydroquinone and related derivatives have been carried out using the density functional theory (DFT).<sup>23,24</sup> The calculations were performed with the Gaussian 03 molecular package<sup>25</sup> and initially all structures were submitted to a PM3 conformational search.<sup>26</sup> Additionally, the Becke's three parameter exact exchange functional (B3) combined with the non-local gradient corrected correlation functional of Lee-Yang-Parr (LYP), denoted as B3LYP,<sup>27-30</sup> was used with the 6-31G\* basis sets<sup>31-34</sup> for an ultimate geometry optimization. The final optimized structures were confirmed to be real minima by frequency calculation (no imaginary frequency). The conformers with the lowest electronic energies were used in this work. The ionization potential (IP<sub>1</sub>) for all molecules was calculated as the energy difference between the cation free radical and its respective neutral molecule (equation 1). In addition, the IP<sub>2</sub> for the ionized molecules were calculated as the energy difference between the semiquinone free radical and its respective anion molecule (equation 2).

$$IP_1 = EPhH^{+} - EPhH \quad (1)$$

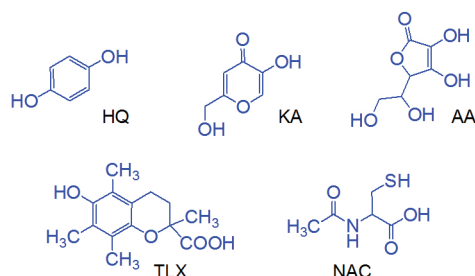
$$IP_2 = EPh^{\bullet} - EPh^{-} \quad (2)$$

The bond dissociation energy of the O-H group ( $BDE_{OH}$ ) and its formation were calculated as the energy difference between the semiquinone form plus the hydrogen radical and the respective neutral molecule (equation 3).

$$BDE_{OH} = (EPh^{\bullet} + EH^{\bullet}) - EPhH \quad (3)$$

Since our interest is to understand the role played by the possible action mechanism of the hydroquinone

oxidation and related derivatives (Scheme 1), we adopted a systematic study comparing the electronic properties for all compounds. In order to achieve this aim, we calculated the following properties: (i) highest occupied molecular orbital (HOMO); (ii) lowest unoccupied molecular orbital (LUMO); (iii) ionization potential (IP); (iv) phenol or enol bond dissociation energy ( $BDE_{OH}$ ). The chemical stability was related with (v) spin densities distribution.



**Scheme 1.** Chemical structures of the hydroquinone, related derivatives, and antioxidants.

## Results and Discussion

### Chemical stability

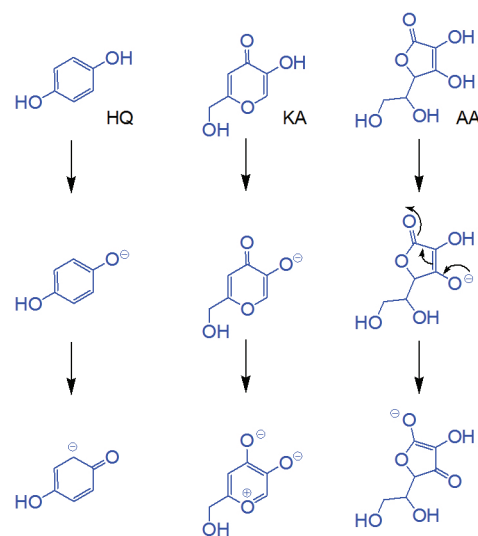
In the present study, the chemical stability of hydroquinone and related derivatives were evaluated by different experimental models and the results show that pH can play a significant role in the oxidation and polymerization processes.

The alterations on formulation of kojic and ascorbic acids on cream or gel in concentration of 5% were insignificant. Moreover, the same formulation of hydroquinone in concentration of 5% showed significant modifications during the formulation process observed by the color changes of gel or cream. Nonetheless, the formulation of hydroquinone in concentration of 5% plus *N*-acetylcysteine association at 0.05% did not show significant alterations.

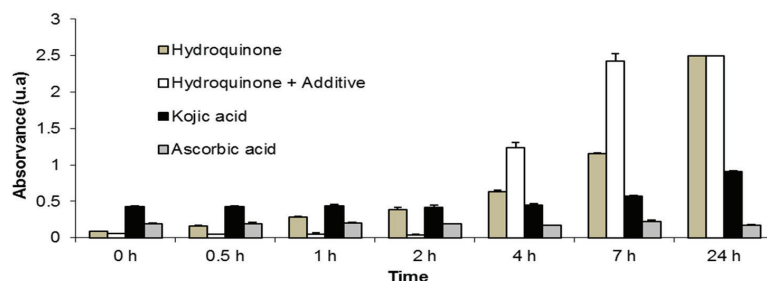
The quantification of this effect was observed in solutions using the same concentrations. However,

no alteration was observed in water or water:ethanol. Nevertheless, the oxidation and polymerization reactions were accelerated by alkalization using sodium bicarbonate 0.05% solution (water:ethanol 1:1)<sup>20</sup> and these results can be seen in Figure 1.

The Figure 1 shows that hydroquinone produced a dose-related oxidation. The kojic acid shows weak polymerization property on the alkaline-induced oxidation. The same property was observed for ascorbic acid. The high chemistry stability of ascorbic acid is related to its resonance effects between enolic and carbonyl moieties. Moreover, the high chemical stability of kojic acid is related to its resonance effects between ether and carbonyl moieties. However, the hydroquinone anion has its negative charge on centered-carbon atom. So, this compound may interfere with the polymerization process after electron abstraction due to the free radical carbon-centered. Nevertheless, the addition of *N*-acetylcysteine at 0.05% in hydroquinone solution has a significant polymerization that starts at the time of 2 h. These resonance structures can be seen in Scheme 2.



**Scheme 2.** Resonance structures of the hydroquinone and related derivatives.

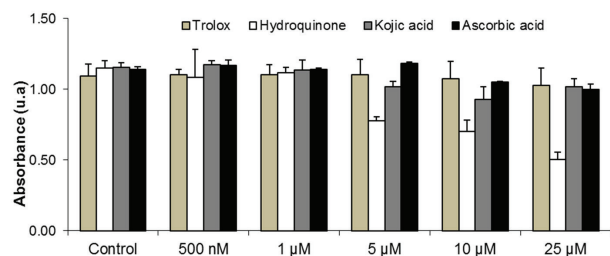


**Figure 1.** Chemistry stability for the hydroquinone, hydroquinone + additive (NAC), kojic acid, and ascorbic acid in alkaline solution (water:ethanol 1:1, sodium bicarbonate 0.05%).  $p < 0.01$  vs. control (0 h), (ANOVA, Tukey's range test).

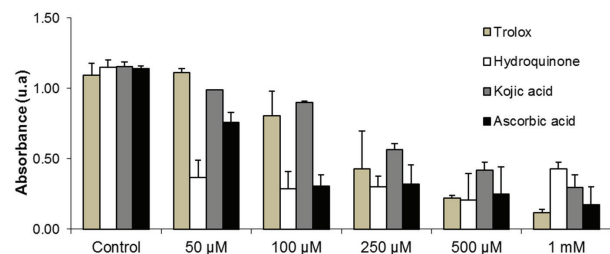
## Antioxidant evaluation

Since this oxidation property may be related to the redox ability of these compounds an antioxidant evaluation was realized. For the antioxidant evaluation several doses were used to assess the profile of these molecules in the reaction against the DPPH free-radical. Trolox was used as standard antioxidant.

The standard antioxidant was able to prevent the increase of inhibition after 100  $\mu\text{M}$ . Similarly, the same behavior was obtained by the kojic acid, which showed a dose-dependence effect starting around of 100  $\mu\text{M}$ . Nevertheless, the antioxidant effect for the ascorbic acid starting on 50  $\mu\text{M}$  and hydroquinone showed a good DPPH inhibition from 5  $\mu\text{M}$  (Figures 2 and 3).



**Figure 2.** Antioxidant evaluation of the hydroquinone, kojic acid, ascorbic acid, and trolox using DPPH radical.



**Figure 3.** Antioxidant evaluation of the hydroquinone, kojic acid, ascorbic acid, and trolox using DPPH radical.

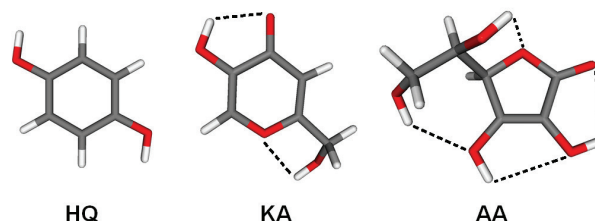
The observed performance for these molecules can be attributed to the numbers and the positions of the hydroxyl phenolic or enolic moieties. Thus, the order of activity is the following: hydroquinone > ascorbic acid > trolox > kojic acid. In fact, hydroquinone and ascorbic acid were the more active for DPPH inhibition and these molecules have two hydroxyl groups. Moreover, trolox is more active than kojic acid considering the chelation between hydroxyl and carbonyl for the kojic and ascorbic acids. The results observed here agree with experimental methods for ascorbic and kojic acids.<sup>35,36</sup>

In fact, our results indicate that a relationship between the oxidation potential and chemical stability can occur. This relationship between the chemical stability and antioxidant properties can be better understood by studying

the structure-property relationship using theoretical methods. For this purpose, we adopted a systematic study comparing electronic parameters of these molecules with experimental properties.

## Structure-property relationship

The chemical stability study of drug candidates should go beyond the design, but it should be present in each step of design and development. In order to understand the chemical stability and the antioxidant mechanisms, all reactions were related to the chemical reactivity of the studied molecules. Thus, a comparative study between structure and chemical reactivity was performed using theoretical methods. First, a conformational search was conducted to select the more stable conformations. The optimized geometries attained through DFT calculations with B3LYP 6-31+G(d,p) basis sets are described in Figure 4. The most stable conformations have several intramolecular hydrogen bonds among enol, alcohol, keto, ether moieties mainly in the kojic and ascorbic acids.



**Figure 4.** Structure of the hydroquinone and related derivatives.

From the structural and electronic analysis of these molecules, it is possible to determine the relationship between chemical reactivity and the influence of each chemical group on the oxidation mechanism. Considering that the oxidation mechanisms of these molecules are known by electron or hydrogen transfer.<sup>37-39</sup> Perhaps the nucleophilicity depends on the redox property of these molecules, mainly from their electron-donating or electron-accepting groups. Therefore, the high stabilization capacity makes them more reactive and stronger nucleophiles. The HOMO, LUMO, IPs, and BDE<sub>OH</sub> of these molecules can be compared for the mechanism elucidation by electron transfer (Table 1).

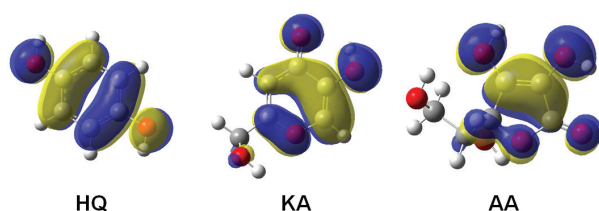
The observed performance for these molecules using electron transfer mechanism can be also attributed to numbers and the positions of the hydroxyl group for the phenolic or enolic moieties. The standard molecule (trolox) showed the highest HOMO value (−5.39 eV) and the lowest LUMO value (−0.51 eV). This molecule has the lowest HOMO-LUMO value gap (GAP, 4.48 eV) and IP (686.15 kcal mol<sup>−1</sup>). In fact, hydroxyl moiety has great influence on molecular orbital. In

**Table 1.** Calculated properties related to redox capacity of the compounds

Compound	HOMO / eV	LUMO / eV	GAP / eV	IP <sub>1</sub> / (kJ mol <sup>-1</sup> )	IP <sub>2</sub> / (kJ mol <sup>-1</sup> )	BDE <sub>OH</sub> / (kJ mol <sup>-1</sup> )
HQ	-5.82	-0.62	5.19	761.98	177.12	354.38
KA	-6.88	-1.91	4.96	867.79	248.19	404.88
AA	-6.93	-1.38	5.54	869.53	303.47	334.54
TLX	-5.39	-0.51	4.48	686.15	203.53	376.70
NAC	-7.26	-0.84	6.42	883.09	276.57	374.89

HQ: hydroquinone; KA: kojic acid; AA: ascorbic acid; TLX: trolox; NAC: *N*-acetylcysteine; HOMO: highest occupied molecular orbital; LUMO: lowest unoccupied molecular orbital; GAP: HOMO-LUMO value gap; IP: ionization potential; BDEOH: phenol or enol bond dissociation energy.

Figure 5 is observed an excessive participation of hydroxyl groups on nucleophilic position of HOMO for hydroquinone, kojic acid, and ascorbic acid.

**Figure 5.** HOMO of the hydroquinone and related derivatives.

In general, the order of activity based on electron transfer is the following: hydroquinone > kojic acid > ascorbic acid. However, the anion forms have many influences on the IP values in acid molecules at alkaline solutions. Nevertheless, no alteration in the activity occurs after the proton dissociation based on IP<sub>2</sub>. On the contrary, the observed performance for these molecules using hydrogen transfer mechanism (BDE<sub>OH</sub> values in kcal mol<sup>-1</sup>) of the hydroxyl group from the phenolic or enolic positions presents the following sequence: ascorbic acid (334.54) > hydroquinone (354.38) > kojic acid (404.88). These results are in accordance with theoretical methods for ascorbic and kojic acids.<sup>40,41</sup> In fact, hydroquinone and ascorbic acid were the most active for DPPH inhibition and these molecules have two hydroxyl groups. Furthermore, trolox is more active than kojic acid. The possible chelation between the hydroxyl and the carbonyl of the kojic acid and the ascorbic acid explains the highest activity of the hydroquinone.

The relationship between theoretical calculation and chemical stability for the hydroquinone formulation using *N*-acetylcysteine cannot be related by combination of an antioxidant that has higher theoretical values, such as HOMO (-7.26 eV), GAP (6.42 eV), IP (883.09 and 276.57 kcal mol<sup>-1</sup>), and BDE<sub>OH</sub> (374.89 kcal mol<sup>-1</sup>) when compared with hydroquinone. Therefore the chemo-protective effect of *N*-acetylcysteine can be related due to polarizability or chelation properties of sulfur moiety. In fact, the administration of sulfhydryl aminoacid (cysteine

or methionine) has been shown to increase the efficacy of chelating agents in the treatment of metal intoxication.<sup>42</sup> Hence, the chemo-protection of *N*-acetylcysteine on hydroquinone formulation is not a classical effect of electron or hydrogen transfers.

## Conclusions

The chemical stability of hydroquinone (HQ) was compared with kojic (KA) acid, antioxidant additives such as ascorbic acid (AA) and *N*-acetylcysteine (NAC) and related to their redox effects. The order for oxidation by hydrogen transfer is AA > HQ > KA, where HQ is more reactive than the other compounds when compared to electron transfer. A synergistic antioxidant protection of NAC was observed on HQ oxidation when compared to AA. DFT calculation with the B3LYP functional did not explain the antioxidant performance for the NAC. The interactions between hydroquinone and NAC can be related to their chemical properties such as polarizability or chelation capacity of sulfur moiety.

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