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Scientific paper

Harnessing Synergy: Investigation of the Antioxidant Interactions Between *trans*-Resveratrol and *L*-Ascorbic Acid by Spectroscopic and DFT Methods

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Abstract

The present study has employed a combination of UV-Vis, ^{13}C NMR spectroscopic and computational methods to explore the antioxidant interactions between *trans*-resveratrol and *L*-ascorbic acid in the reaction with 2,2-diphenyl-1-picrylhydrazyl radical. Significant synergic and antagonistic effects were observed depending on the reaction conditions. Various molar ratios of antioxidants have been tested, and the highest synergistic interaction has been registered for the 1:1 ratio of antioxidants. ^{13}C NMR spectral data argued on the dimerization of resveratrol producing the known natural *trans*-*δ*-viniferin as a crucial phenomenon involved in the synergetic antioxidant activity. The Density Functional Theory data completed the research, advancing the possibility of synergistic interactions through the regeneration of resveratrol's and its oligomer's radicals by the ascorbic acid *via* the hydrogen atom transfer pathway.

Keywords: *Trans*-resveratrol, ascorbic acid, *trans*-*δ*-viniferin, UV-Vis, ^{13}C NMR, DFT, synergism

1. Introduction

From the class of natural phenolic compounds, *trans*-resveratrol (tRes) (Figure 1) is one of the well-known representatives of stilbenoids that are widely spread in fruits, vegetables and legumes, the fruits with the highest content of it being grapes.[1] This compound owes its fame to a plethora of biological properties, *e.g.* antioxidant,[2] antimicrobial,[3] cardioprotective[4] and antitumor[5–7] that also explains why it has been intensively investigated in the past years. The latest data prove tRes to be one of the most eminent anti-age vehicle that prevents dendritic cell maturation in response to advanced glycation end products,[8] regulates inflammation including some of severe inflammatory neurodegenerative processes, and improves oxidative stress *via* Nrf2 signalling pathway.[9–10]

Recent studies attested the powerful ability of tRes to scavenge free radicals using different tests and a mechanism of antioxidant activity of tRes was proposed relying on the electron delocalization between the two phenolic rings to give the tRes-quinone structure.[11,12] These considerations do not contradict the hypothesized biosynthetic pathways involved in oligomerization of tRes.[13]

Viniferins are natural tRes-derived metabolites, commonly found in many food products, with grapes (*Vitis Vinifera*) serving as a primary source.[5] Amongst the most widespread in natural sources there are some tRes dimers, such as *ε*-viniferin, *trans*-*δ*-viniferin (Figure 1), and trimers, *e.g. α*-viniferin.[5]

The manifold biological activities are reported for them, including anti-inflammatory,[14] anti-cancer,[15] neuroprotective,[16] anti-diabetic,[17] anti-microbial,[18] as well as marked antioxidant effects,[19–21] xanthine oxidase inhibition properties[22–24] and targeting DNA duplex and G-quadruplex structures, by this interfering with the new appealing strategies for focused anticancer therapies.[24,25]

It is worth mentioning that oligomers of tRes and tRes itself are known as phytoalexins produced by many plants, having strong implication in their antioxidative defence system in response to infection, UV-irradiation or

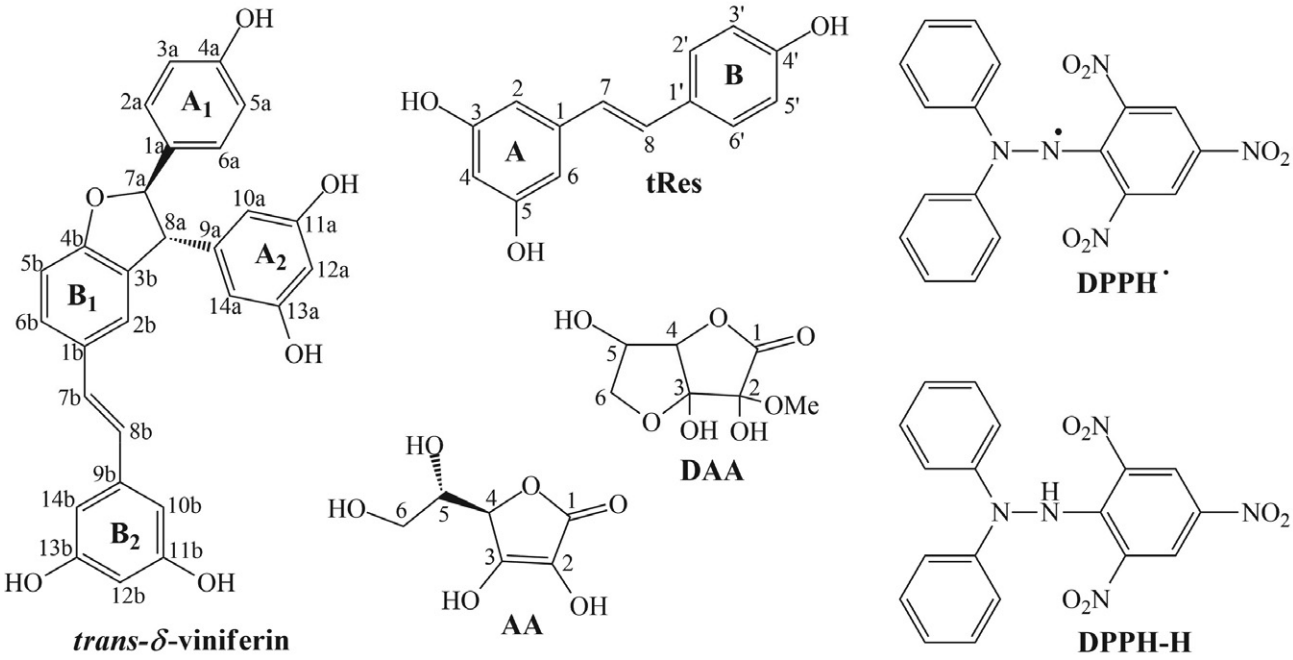

Figure 1. Chemical structures of *trans*-resveratrol (tRes); *trans*-δ-viniferin; *L*-ascorbic acid (AA); the enantiomers of bicyclic dehydroascorbic acid solvated at C-2 (DAA); 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•); and the reduced form of DPPH radical (DPPH-H).

other types of physiological stimuli.[5,13,27] Structural characterization, including determination of the absolute configuration, and their naming are still an area of confusion, in virtue of the presence of two stereo-chemical centres at positions 7a and 8a on the dihydrofuran ring and the presence of *trans* (E) or *cis* (Z) double bond (Figure 1).

Due to significant biological properties of tRes and viniferins, the interest of chemists is maintained vivid and various approaches of chemical oxidation of tRes have been reported, producing its oxidized derivatives.[23,27–29] In the light of the afore-mentioned, further elucidation, regarding both the palette of biological effects and mechanisms governing and explaining the processes accompanying the conversions of tRes are welcome.

As a part of our studies on antioxidants naturally found in grape, we have concentrated our attention to the antioxidant's interactions (AI),[30,31] since studies on various foods, plants and extracts or singular compounds have demonstrated that there may be a significant discrepancy between the antioxidant activity of a complex mixture and the sum of the antioxidant activities of its constituent compounds, which can be synergistic, additive or antagonistic.[32,33] According to Tsao R, the three types of antioxidant interactions are defined as follows: (1) synergistic antioxidant interaction – the antioxidant effect of two or more discrete antioxidants when applied together is greater than the sum of the individual antioxidant effects applied separately; (2) additive antioxidant interaction – the antioxidant effect of two or more discrete antioxidants when applied together is equal to the sum of the individual antioxidant effects applied separately; (3) antagonistic antioxidant interaction – the antioxidant effect of two or more discrete antioxidants when applied together is less than the sum of the individual antioxidant effects applied separately.[32]

To our knowledge, few studies have investigated the antioxidant interactions between *trans*-resveratrol and *L*-ascorbic acid (AA) (Figure 1), despite the fact that both compounds are present in grapes and wines[34], and could easily interact. AA is among the most explored water-soluble natural antioxidants, being endowed with powerful free radical scavenging activity. Since AA has been proved to act as a primary scavenger of radical species, it is frequently used as a reference compound when studying the antioxidant activity of other active species.

In wines, AA is mostly present from adding it during the white wine production, particularly just prior to bottling,[35] since it complements SO_2 as an antioxidant in wine, by this helping to preserve desirable fruit characters during wine development.[35] Recent outcomes give evidence about the similar antioxidant benefits provided by ascorbic acid for rosé wine as it does for white wine.[36] At the same time, the higher concentrations of phenolic compounds in red wines are reported to negate the need for further antioxidant addition.[34]

Only few data were found in the literature regarding the assessment of reciprocal interactions for mixtures comprising tRes and AA by using the antioxidant test with DPPH• (Figure 1),[37] though DPPH assay is a popular method to determine the antioxidant and antiradical ac-

tivity of a substrate, being usually measured on a common UV-Vis spectrophotometer.[38–40] It should be mentioned that, the reaction of tRes with DPPH• constituted a part of the thorough investigation regarding the radical-scavenging activities and mechanisms of tRes itself and tRes-oriented analogues, comprising the study of the influence of solvent, radical, and substitution.[29]

It is worth mentioning that tRes and some of its oligomers have shown comparable antioxidant activity in the DPPH, FRAP and NO-scavenging assays by UV-Vis spectroscopy.[21] Recent studies have also demonstrated the enhancement of the antioxidant activity in the binary and ternary mixtures of tRes and viniferins, caused by the synergistic interactions.[21]

^{13}C nuclear magnetic resonance (NMR) spectroscopy has been documented as an efficient tool for clarification of the molecular mechanisms underlying the antioxidative and radical-scavenging activities of tea polyphenols.[41] This method offered a more detailed data on the structural changes that occurred to the tea antioxidants molecules after interaction with DPPH radical.

The current study portrays the tRes – AA system in an attempt to model and elucidate the plausible interactions between antioxidants that are naturally found in grape. The interplay between antioxidants in the investigated system is suggested by using the DPPH assay and relying on the data of UV-Vis and ^{13}C NMR spectroscopies as methods of choice. The obtained data are discussed through the prism of the available data from the literature, while the application of Density Functional Theory (DFT) method offers some additional mechanistic insights, with regard to the plausible molecular interactions in the analysed system of antioxidants.

2. Experimental

2. 1. Materials

Commercial reagents: *trans*-resveratrol (tRes; >98.0%, Sigma-Aldrich, USA), *L*-ascorbic acid (AA; 99.0%, Sigma-Aldrich, Germany), *L*-dehydroascorbic acid (DAA; Sigma-Aldrich, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH•; Sigma-Aldrich, Germany); acetone-d_6 (99.9 atom % D, contains 0.03% (v/v) TMS; Sigma-Aldrich, USA); methanol-d_4 (99 atom % D, Sigma-Aldrich, USA), ethanol (EtOH; 96%, MicTan, Republic of Moldova) were used without any additional purification for UV-Vis and NMR experiments.

2. 2. Apparatus

Absorbance measurements were recorded on a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer), at 25 °C, using 10 mm quartz cuvettes. NMR spectra were recorded on a Bruker AVANCE 400 spectrometer equipped with a 5-mm broadband reverse probe with field z gradient, operating at 100.61 MHz for ^{13}C nuclei, at 25 °C. For ^{13}C spectra 10240 scans were registered. Chemical shift (δ) are reported in parts per million (ppm) and are referenced to the residual non-deuterated peak of methanol-d_4 (49.0 ppm).

2. 3. UV-Vis Spectrometric Measurements

tRes and AA were dissolved in 96% EtOH to obtain 1.14 mM of each solution, being further used in the experiments. The concentration of DPPH• in 96% EtOH was verified daily though the calibration line and was around 1.600 ± 0.020 a.u. The absorption maximum of DPPH• was found at 517 nm, with a molar extinction coefficient, ε, of 11858 ± 16 $M^{-1}cm^{-1}$.[30,38]

The antioxidant activity was estimated by using slightly modified procedure described by Brand-Williams.[42] To 3.8 mL of free radical, 0.1 mL of tRes or AA solution was added, along with 0.1 mL EtOH in order to determine the antioxidant activity of single compounds. To establish the antioxidant activity of the tRes – AA mixtures against DPPH•, three approaches of performing the reactions were followed: 1st) mixing 0.1 mL of each solution of antioxidants in the 1:1 tRes/AA molar ratio, followed by the addition of 3.8 mL DPPH• (reaction time – 1 hr); 2nd) mixing 0.1 mL tRes and 3.8 mL DPPH• (reaction time – 1 hr), then adding 0.1 mL AA (reaction time – 15 min); and 3rd) mixing 0.1 mL AA and 3.8 mL DPPH• (reaction time – 15 min), then adding 0.1 mL tRes (reaction time – 1 hr). The reactions were carried out in 96% ethanol.

Furthermore, to evaluate the influence of the concentration of tRes and AA on the antioxidant activity of the binary combinations, several tRes/AA molar ratios, as it follows: 1:5, 1:4, 1:3, 1:2, 1:1.5, 1:1, 1.5:1, 2:1, 3:1, 4:1 and 5:1 (in these assays, the second term of the ratio denotes the dilution relative to the other solution), have been investigated, using the same procedure described above.[42] The impact of the tRes/AA molar ratio on the antioxidant activity was evaluated following the 2nd approach of performing the experiment. The blank reference cuvette contained 96% ethanol. All measurements were performed at least in triplicate.

The data analysis was performed following the reported methods.[39,40] First, the percentage of DPPH• inhibition (%Inhibition) was calculated using equation 1,[39,40] the values obtained being further used to determine the AI type.

$$\%\text{Inhibition} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad (1)$$

where, A_{sample} is the absorbance of the sample at steady state and $A_{control}$ is the absorbance of the sample at time zero.

The AI effect of a mixture was calculated from the ratio between the experimental value of the percent of

DPPH• inhibition of the mixture (%I*e*) and the theoretical value (%I*t*),[39,40] as follows:

$$\mathrm{AI} = \frac{\%I_e}{\%I_t} \quad (2)$$

where

$$\%\mathrm{I}_{\mathrm{theoretical}} = \%I_A + \%I_{tRes} - \left(\frac{\%I_A \times \%I_{tRes}}{100}\right) \quad (3)$$

$\%I_A$ and $\%I_{tRes}$ represent the percent inhibition of each antioxidant (ascorbic acid and *trans*-resveratrol, respectively), tested in reaction with DPPH• (equation 3).

According to the reported methods,[39,40] a synergistic effect is found when the AI > 1; if AI = 1, then the interaction is additive; and an AI < 1 reveals an antagonistic effect.

Data obtained were analysed with ANOVA and Student's t tests to evaluate the statistical significance of the difference between the means using the Microsoft Excel programme. A *p* value of 0.05 was considered significant.

2. 4. NMR Spectrometric Measurements

In the ^{13}C NMR experiments, the reactions of AA and tRes with DPPH• were carried out in the mixture of deuterated solvents acetone-d_6 – methanol-d_4 in the NMR tube. The AA : tRes : DPPH• (1:1:2.6) molar ratios were mainly analysed. The solutions for analysis of single compounds consisted of 0.015 mmol of tRes or AA, and 0.039 mmol for DPPH• in a total solvent volume of 0.7 mL. The AI between tRes and AA were investigated by following two approaches[41]: 1st) mixing antioxidants and free radical (reaction time – 1 hr), NMR analysis; 2nd) mixing tRes and free radical (reaction time – 1 hr), then adding AA (reaction time – 15 min), followed by NMR analysis.

2. 5. Theoretical Method Details

In this research, the molecules of interest (AA, tRes, DPPH•) were optimized using ORCA 5.0 software.[43] The optimized structures of the respective neutral molecules, radicals, radical cations, and anions were confirmed as true minima through frequency calculations at the same level of theory (lack of any imaginary frequency). The energies of the above mentioned species were calculated by DFT method using the 6-311++G(d,p) basis set and the Becke, 3-parameter, Lee–Yang–Parr (B3LYP) functional, with the aim of calculating the bond dissociation energy (BDE), adiabatic ionization potential (AIP), proton dissociation energy (PDE), proton affinity (PA), electron transfer energy (ETE), and the Gibbs Free Energy of the reactions (ΔrG0). Dispersion correction was done by using Grimme's DFT-D3 approach with the newer recommended Becke-Johnson damping (D3BJ).[43] All calculations have been carried out in ethanol, using the SMD solvation model.[44]

3. Results and Discussion

3. 1. UV-Vis data

Spectrometric methods, including DPPH assay, have been widely used in the determination of the antioxidant activity of pure compounds and complex mixtures.[38–40] Polyphenols are well recognized for their prominent antioxidant properties,[45] our studies regarding antioxidative effects of quercetin[46] and proanthocyanidins[47] representing some of our contributions to this topic.

As reported in the literature, the abilities of tRes and AA to act as free radical scavengers are different: AA possesses stronger than tRes antioxidant activity, therefore, to annihilate a mole of DPPH• 1 mole of tRes is required, and only 0.24 mole of AA.[38,48]

The AI of tRes or AA in combination with various antioxidant and non – antioxidant compounds have been described,[33,49–51] the available evidence indicating about some factors that do significantly influence AI, such as: the used method, concentration of compounds, solvent, reaction conditions etc.[33,52]

Our research has been conducted in order to gain further insight into the problem of interactivities for the tRes - AA system of antioxidants, as follows. As afore-discussed, the ability of AA to quench the DPPH• is more elevated than of tRes, by this explaining its higher antioxidant activity.[38] Therefore, it seemed appropriate to clarify first of all, the type of AI of the mixture tRes – AA in reaction with DPPH•, as a function of the consecutiveness of incorporation of antioxidants. The AA : tRes = 1:1 reaction stoichiometry has been used. The AI has been calculated based on the UV-Vis measurements.

The results of these studies are depicted in Figure 2. As demonstrated by experimental data, only one approach proved evidence in favour of synergistic AI, while the other two argued about the antioxidant's antagonism. As it can be noticed from Figure 2, only the 2nd approach, *i.e.* the case when DPPH• assisted conversion of tRes first occurs, succeeded by the addition of AA, offered a high synergistic effect of 1.19. Compared to the 2nd approach, the 1st (simultaneous interaction of tRes and AA with free radical) and 3rd (addition of tRes to the reaction mixture,

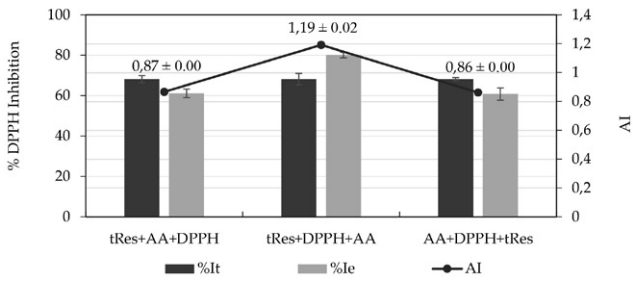

Figure 2. Graphical representation of the Percentage of theoretic (%I*t*) and experimental inhibition of DPPH• (%I*e*), and AI for the 1st, 2nd and 3rd approach, correspondingly. Data are presented as mean values (n ≥ 3). Significant difference ($p < 0.05$) between AI values calculated using one way ANOVA test.

preceded by interaction of DPPH• with AA) procedures have the experimental percentage values of DPPH radical inhibition (%I*e*) lower than the theoretic ones (%I*t*), by this testifying pro about the antagonistic type of AI of 0.87 and 0.86, respectively for these two cases.

By the obtained results one can state that upon interaction with DPPH•, a critical factor for a specific AI to occur is the consecutiveness of adding the antioxidants to radical, since both synergistic and antagonistic AI between tRes and AA has been attested. Moreover, based on this observation, one can firmly conclude about the role of tRes-derived compounds obtained upon its interaction with DPPH• in manifestation of synergism. As emerges by comparison of all three experimental routes, since AA is faster in scavenging the free radical,[38] the 3rd and 1st approaches offer almost the same AI effect, while only in the 2nd case, mixing AA to the already converted by DPPH• tRes produces the synergic effect. Accordingly, changing the sequence of interaction between the antioxidants and free radical finds expression in the outcome of the overall process, by this reflecting the different mechanisms of AI.

Further, the impact of the molar ratio of antioxidants on the antioxidant activity has been investigated by UV-Vis method and for this reason various tRes/AA molar ratios have been assayed employing the DPPH• test, respecting the 2nd approach of performing the experiment. As depicted in Figure 3, all samples with various tRes/AA molar ratios, starting from 5:1 to 1:5, have demonstrated synergistic effect. The strongest synergy, found as a value of 1.19 have been registered for the 1:1 tRes/AA molar ratio, followed by 2:1 and 1:2 ratios with synergistic effects of 1.17 each. The 1.5:1 and 1:1.5 tRes/AA molar ratios registered an AI of 1.14. As the difference in concentration between the two antioxidants becomes greater, the synergistic effect decreases (Figure 3). The lowest AI of 1.09 was noticed for the 1:4 tRes/AA molar ratio.

The synergistic AI are of most interest for science and industry due to the advantages that they can offer.[32] Therefore, our further investigations employing NMR have been focused on the establishment of the action mechanism behind the synergistic effect of the 1:1 tRes/AA molar ratio (2nd approach), particularly by mean of ^{13}C NMR.

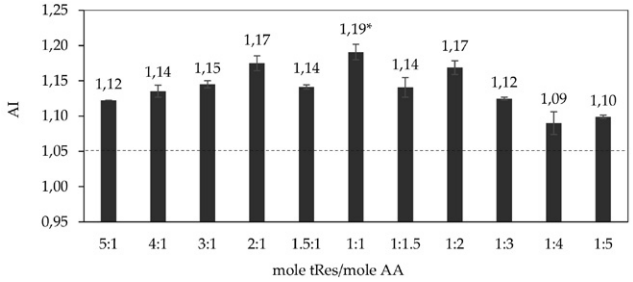

Figure 3. Graphical representation of AI for various molar ratios of mixtures tRes/AA. The values above 1.05 (the dashed line), inclusively, describe the synergistic AI. Data are presented as mean values (n ≥ 3). Significant difference ($p < 0.05$) calculated using one way ANOVA test. *Significant difference ($p < 0.05$) to 1.19 calculated using Student's t test.

3. 2. NMR Data

For many years, NMR spectroscopy remains one of the analytical methods that is essential for wine authentication.[53] The successful use of NMR for investigating the molecular mechanisms of the radical scavenging activities of antioxidants in tea[41] and fruits[54] has been described. By characterizing the changes in the ^{13}C NMR spectra, the authors unveiled the structural changes of antioxidants after scavenging the DPPH radicals at the molecular level, by this determining the active site of each antioxidant in each radical-scavenging reaction and comparing the relative radical-scavenging activity between antioxidants, as well.[41]

NMR spectral data have been reported for structure identification of various viniferins,[28,29,55,56] while ^{1}H (1D and 2D homonuclear ^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY proved to be particularly useful for assignment of hydrogen nuclei and establishment of the stereochemical configuration, e.g. for (+)-*α*-viniferin.[57]

In the ^{13}C NMR studies that we have carried out with the aim of shedding light on the interactions between the studied antioxidants, the 1st and 2nd approaches of performing the reactions were the subject of a comparative analysis, since by UV spectral method the close AI values were found for the 1st and 3rd approaches (Figure 2).

For modelling the AI in the investigated system, the reaction of AA and tRes with the DPPH radical was carried out in the NMR tubes, in the mixture of acetone-d_6-methanol-d_4 solvents, due to the different solubility of the reactants. The AA : tRes : DPPH•= 1:1:2.6 stoichiometry has been investigated. The chemical shifts for ^{13}C nuclei in AA, DAA and reduced DPPH• (DPPH-H) in our NMR experiments were in accordance with the reported data.[41] In order to facilitate the analysis of the ^{13}C NMR data and to well-differentiate the signals in the ^{13}C NMR spectra, belonging to different species of compounds in the analysed mixtures, the following characteristic peaks have been chosen by us for identification of: AA – 64.3 ppm, DAA – 89.9 ppm, tRes – 117.1 ppm, reduced DPPH• (DPPH-H) – 122.1 ppm, tRes dehydrodimer – 58.9 ppm (Figure 4).

First, according to our results, in the studied acetone-d_6 solution containing tRes – DPPH• reaction, the ^{13}C resonances for the described oligomer of tRes – *trans*-*δ*-viniferin (tRes dehydrodimer) have been detected in the spectrum (Figure 4, case A), confirming the known data.[29,58–60] It should be mentioned that even though most of the ^{13}C NMR signals for resveratrol dehydrodimer overlap with the signals of tRes itself, the resonance at δ 58.8 ppm that has been described as typical for 8a carbon nucleus of *trans*-*δ*-viniferin[27,61] (Figure 1) undoubtedly attest its presence in solution (Figure 4, case A).

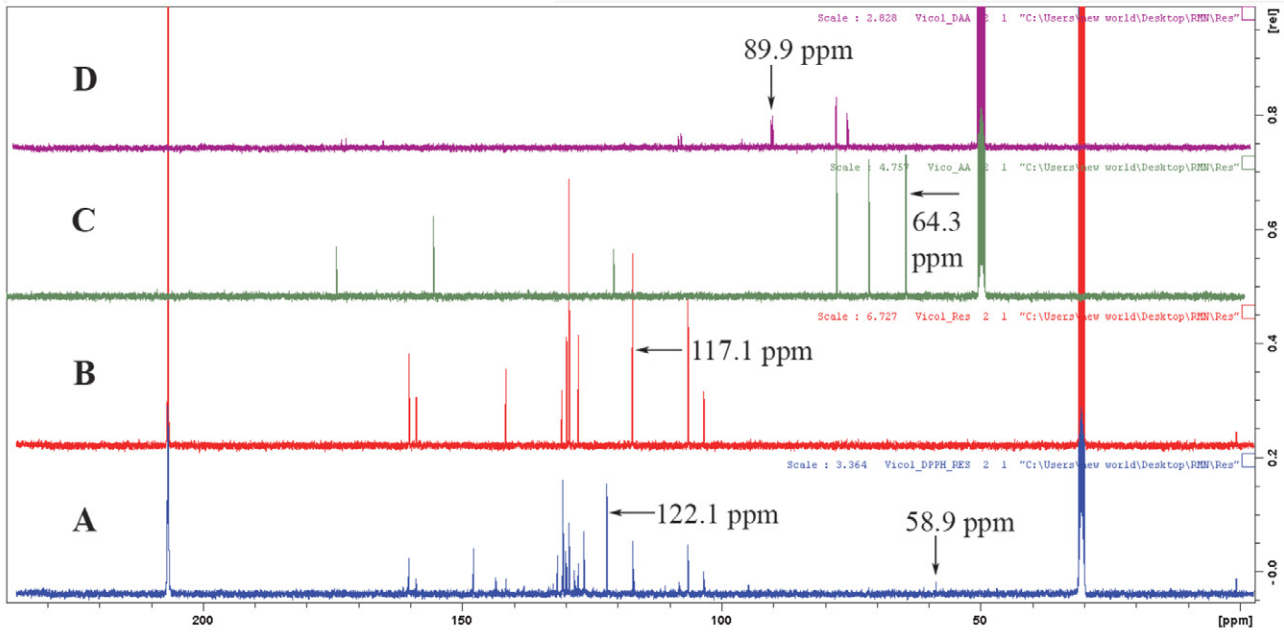

Figure 4. ^{13}C NMR spectra: A) reaction of tRes with DPPH$^{\bullet}$, 1:1 molar ratio, r.t., 1hr., in acetone-d_6; B) tRes in acetone-d_6; C) AA in methanol-d_4; D) DAA in methanol-d_4.

It is important to mention about the available literature reports containing the full ^{13}C NMR characteristics for *trans*-δ-viniferin, in which particularly the formation of it as the main reaction product of tRes oxidation is outlined. Thus, Shang *et al.* describe its preparation under the name 'dimer of resveratrol' upon oxidative conversion of tRes in the presence of galvinoxyl radical in ethanol[29] and Wang *et al.* have identified it as major compound upon analysis of radical reaction product of DPPH$^{\bullet}$ and tRes.[58] Mei *et al.* have reviewed a series of articles describing the *in vitro* oxidation of tRes producing *trans*-δ-viniferin by following biocatalysis and biotransformation of tRes in microorganisms.[28] Breuil *et al.* present the ^{13}C NMR characterization for the resveratrol *trans*-dehydrodimer, after metabolization of tRes by a laccase-like stilbene-oxidase of *Botrytis cinerea*, the causal fungus for grey mould,[59] while Pezet *et al.* identified *trans*-δ-viniferin (synonym to *trans*-resveratrol dehydrodimer) in grapevine leaves infected by *Plasmopara viticola* (downy mildew) or UV irradiated as one of the most important phytoalexins derived

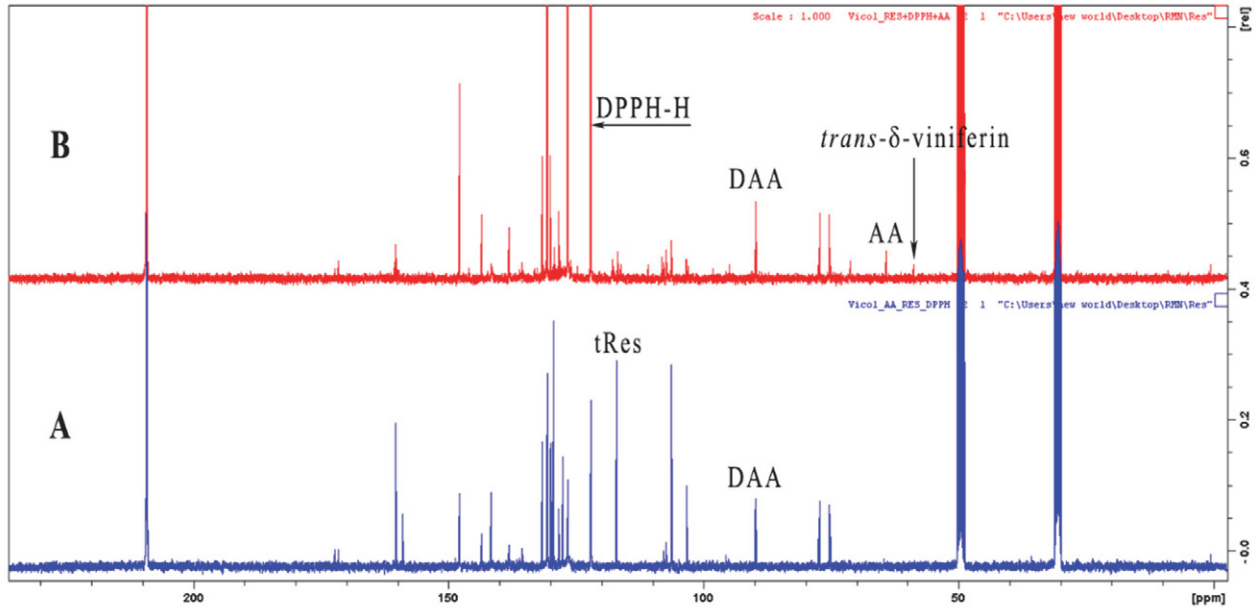

Figure 5. ^{13}C NMR spectra: A) AA : tRes : DPPH$^{\bullet}$ (1:1:2.6) molar ratio, 1st approach; B) tRes : DPPH$^{\bullet}$: AA (1:2.6:1) molar ratio, 2nd approach.

from tRes.[60] The cited sources[28,58–60] present detailed physico-chemical characteristics for the discussed tRes dimer, including ^{1}H and ^{13}C NMR data, without mentioning about the stereochemistry at 7a and 8a carbon atoms.

The chemical shifts for the ^{13}C nuclei distinguishing *trans*-δ-viniferin in the experimental NMR spectrum (Figure 5, case B) are in agreement with the previously published data (for commodity, only the well-separated by other picks signal at δ 58.8 ppm is stressed).[27,61] It should be mentioned, that the reported in the literature use of 2D NMR spectroscopic analyses, including ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{1}H-^{1}H COSY, and ^{1}H-^{1}H NOESY have allowed the full structural elucidation, including establishing of the configuration at the 7a and 8a stereo-centres.[61]

Taking into consideration the afore-mentioned, the hypothesis about the tRes regeneration from its oxidized form by AA, as it was reported for other phenolic compounds[41], must be probably excluded, since polymerization processes are generally known as irreversible.

It is worth underlining that when exploring the AI by mean of the ^{13}C NMR we have found that tRes dimerization occurs only by following the 2nd approach of performing the discussed reaction, for which strong synergistic AI have been established *via* the UV-Vis spectroscopy (Figure 2). In Figure 5, cases A and B, the ^{13}C NMR spectra are presented for comparison, showing both modes of conducting the reaction that help to get insight into synergetic dynamics.

On careful inspection of the spectra in Figure 5, one can notice that upon initial interaction of tRes with the DPPH• followed by ulterior addition of AA, in the ^{13}C NMR spectrum the low intensity signals for AA are found, and for the species of the converted forms of both antioxidants (tRes dehydrodimer and DAA) (case B). This gives evidence for the complete oxidation of tRes upon its interaction with DPPH•, and the subsequent scavenging of the remaining radical species by AA. On the other hand, the investigated by ^{13}C NMR processes by using AA : tRes : DPPH• =1:1:2.6 molar ratio and finding in solution of the discussed different molecular species, including AA (Figure 5, case B) can serve as an additional confirmation of the established by using UV-Vis spectral method reaction stoichiometry.[38] Conversely, on analysis of the simultaneous interaction of both antioxidants with the free radical following the 1st approach (Figure 5, case A), the ^{13}C NMR data corroborate the available from literature data, namely, the AA rapidly scavenge all DPPH•, as it interacts faster, and tRes remains unconverted.

The reactions modelled under the investigated system of antioxidants AA – tRes upon interaction with DPPH• can be considered as mimicking the natural pathways. It was assumed that in nature, the oxidatively generated phenoxyl radicals are mediating the oligomerization of tRes[5,29], producing viniferins, as originally proposed by P. Langcake and R. Pryce.[13]

On the basis of the afore analysed experimental data, we can emphasize that the found synergistic type of interaction in the investigated system of antioxidants tRes and AA by using UV-Vis and ^{13}C NMR spectroscopies as methods of choice, is related to the formation of *trans-δ*-viniferin (Figure 6).

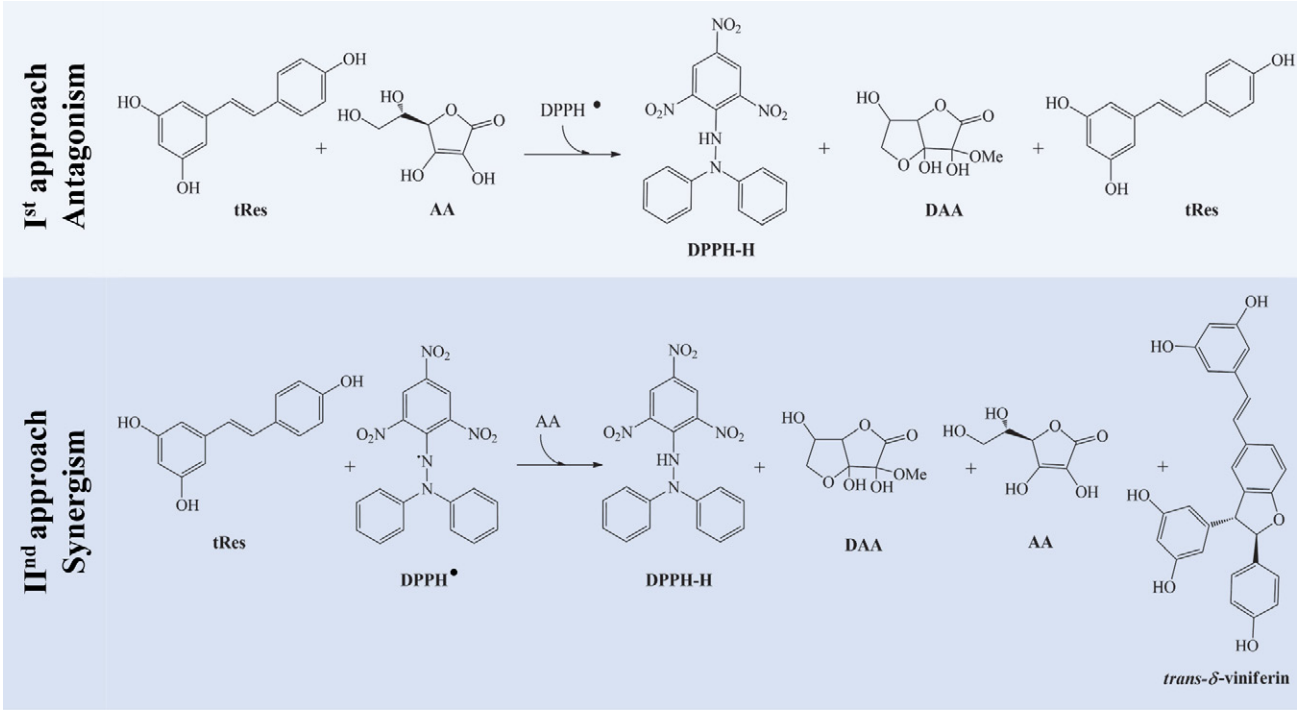

Figure 6. Different reaction products and AI generated under the Ist and IInd approaches of performing the reaction.

Our conclusion is in line with the literature, according to which the formation of dimers with a greater antioxidant power than that of the parent compounds is one of the hypotheses proposed to explain the mechanism of synergistic AI in complex mixture of antioxidants.[33,52] On the other hand, this seem to be in contrast with the reported data on comparison of the scavenging capacity of resveratrol and *δ*-viniferin by using electron paramagnetic resonance spectroscopy.[62] Thus, in reaction with DPPH and NO radicals, *trans*-*δ*-viniferin exhibited lower antioxidant activity than tRes and structurally related compounds like piceatannol and *trans*-piceid. In the •OH model, however, the radical scavenging ability of *trans*-*δ*-viniferin exceeded that of tRes, this finding supporting the idea that stilbenes with higher scavenging capacities for the •OH radical are able to assist the cell in preventing damage.[62]

3. 3. DFT data

To further elucidate the underlying mechanisms of the synergistic interaction between tRes, AA and *trans*-*δ*-viniferin, computational methods such as Density Functional Theory (DFT) can be employed. DFT calculations can provide valuable insights into the electronic structure, energy profiles, and reaction pathways involved in the reactions determining the antioxidant mechanism. By simulating the interaction between the antioxidants and DPPH•, it is possible to identify the thermochemical parameters that describe the different reaction pathways and thus have a better understanding of the observed synergistic effect.

It was previously shown in an extensive series of research dedicated to the quality of wines[63] that dihydroxyfumaric acid, which bears many structural similarities with *L*-ascorbic acid, was responsible for the slow regeneration of the oxidized polyphenols thus enhancing the quality of wines during maturation and their stability. Other studies showed that Vitamin C regenerated green tea polyphenols[64] and Vitamin E.[65,66] For this reason, taking into account the rationale detailed above, it was decided to use computational techniques to explore the following two research hypotheses. The first one refers to synergism as a consequence of the polymerisation processes and formation of more active species. By extrapolating this theory on the actual study, *trans*-*δ*-viniferin should have higher antioxidant activity towards DPPH• than tRes, so its formation would increase the antioxidant potential of the mixture. The second hypothesis addresses the synergism as a mutual regeneration process between the involved antioxidants. Specifically, when AA is added to a reacting mixture of tRes+DPPH•, it would be thermodynamically more feasible to react with tRes radical or with the formed *trans*-*δ*-viniferin radical and regenerate the species. The regenerated tRes and/or *trans*-*δ*-viniferin would then be again included into the process of DPPH• quenching, thus increasing the overall antioxidant activity of the mixture.[47,52,67]

The process of quenching free radicals is mainly explained by the following three antioxidant mechanisms: the hydrogen atom transfer (HAT) mechanism, the single electron transfer proton transfer (SET-PT) mechanism, and the sequential proton loss electron transfer (SPLET) mechanism.[67,68]

The above mechanisms are characterized by the following thermochemical parameters: bond dissociation enthalpy (BDE), adiabatic ionization potential (IP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE).

The numerical values of these parameters are obtained according to the following equations:

$$BDE = H(RO^{\bullet}) + H(H^{\bullet}) - H(R{-}OH) \quad (4)$$

$$IP = H(ROH^{\bullet+}) + H(e^{-}) - H(R{-}OH) \quad (5)$$

$$PDE = H(RO^{\bullet}) + H(H^{+}) - H(ROH^{\bullet+}) \quad (6)$$

$$PA = H(RO^{-}) + H(H^{+}) - H(R{-}OH) \quad (7)$$

$$ETE = H(RO^{\bullet}) + H(e^{-}) - H(RO^{-}) \quad (8)$$

where $H(RO^{\bullet})$ is the enthalpy of AA• or tRes•, respectively, formed after abstracting of H atom from the OH group of the respective compound. $H(H^{\bullet})$ is the enthalpy of a single H atom at the B3LYP/ 6-311G++(d,p) level. H(R–OH) is the enthalpy of the neutral molecule of AA or tRes, respectively. $H(ROH^{\bullet+})$ is the enthalpy of the respective radical cation. $H(e^{-})$ is the enthalpy of single electron. $H(H^{+})$ is the enthalpy of proton. $H(RO^{-})$ is the enthalpy of charged molecule after abstracting of a proton from the OH group. The calculated enthalpies of the hydrogen ($H^{\bullet}$), electron (e^{-}) and proton (H^{+}) in ethanol were taken from the literature.[69–71]

The values obtained for BDE, IP, PDE, PA and ETE (kJ mol^{-1}) for AA, tRes and *trans*-*δ*-viniferin in ethanol are presented in Table 1, where the minimal value shows the preference for a specific antioxidant mechanism. In ethanol, the SPLET mechanism seems to be the most probable for all compounds, with PA values of 168.8, 179.8 and

Table 1. B3LYP/6-311++G(d,p) theoretical O-H bond dissociation enthalpy (BDE), adiabatic ionization potential (IP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE) in ethanol at 298.15 K, for tRes, AA and *trans*-*δ*-viniferin, in kJ mol^{-1}.

Compound	Ethanol				
	HAT	SET-PT		SPLET	
	BDE	IP	PDE	PA	ETE
tRes	322.8	425.9	87.6	179.8	333.7
AA	325.0	505.0	10.6	168.8	345.8
trans-*δ*-viniferin	354.0	420.4	124.3	184.4	360.3

184.4 kJ mol^{-1}, for AA, tRes and *trans*-δ-viniferin, respectively. The SET-PT mechanism is the least probable one, with values of IP of 505.0, 425.9 and 420.4 kJ mol^{-1}, respectively. The BDE values of the three investigated compounds are very similar, of 325.0, 322.8 and 354 kJ mol^{-1}, for AA, tRes and *trans*-δ-viniferin, respectively.

Of course, the three mechanisms described above are competitive, and the most probable one depends on the reaction conditions and nature of the involved free radicals, while polarity of the solvent significantly influence the reaction pathway.

In order to get a more detailed picture of the processes taking place in the reaction mixture, and to explore the first research hypothesis, that *trans*-δ-viniferin has a higher antioxidant activity towards DPPH$^{\bullet}$ than tRes, the half-reactions for DPPH$^{\bullet}$ and the three antioxidant species (AA, tRes and *trans*-δ-viniferin) have been further investigated, according to the following equations:

HAT: $AOX + DPPH^{\bullet} \rightarrow AOX^{\bullet} + DPPH{-}H$ (9)

SET: $AOX + DPPH^{\bullet} \rightarrow AOX^{\bullet+} + DPPH^{-}$ (10)

PL: $AOX + DPPH^{\bullet} \rightarrow AOX^{-} + DPPH^{\bullet+}$ (11)

where AOX denotes one of the antioxidant species, AA, tRes or *trans*-δ-viniferin, respectively.

The values of the Gibbs Free Energy of the reactions ($\Delta_r G^0$) have been calculated according to the formulas:

$$\Delta_r G^0\,(\text{HAT}) = [G(AOX^{\bullet}) + G(DPPH{-}H)] - [G(AOX) + G(DPPH^{\bullet})] \quad (12)$$

$$\Delta_r G^0\,(\text{SET}) = [G(AOX^{\bullet+}) + G(DPPH^{-})] - [G(DPPH^{\bullet}) + G(AA)] \quad (13)$$

$$\Delta_r G^0\,(\text{PL}) = [G(AOX^{-}) + G(DPPH\text{-}H^{\bullet+})] - [G(AOX) + G(DPPH^{\bullet})] \quad (14)$$

Calculation results (Table 2) show that none of the mechanisms is characterized by an exergonic value of the Gibbs Free Energy of the reaction. The HAT mechanism is the most probable one for the reaction of DPPH$^{\bullet}$ with the investigated compounds, because it has the lowest endergonic values of $\Delta_r G^0$. It is also clear that the value of the Gibbs Free Energy of the reaction of DPPH$^{\bullet}$ with tRes is 2.4 kJ mol^{-1}, lower than for the reaction of AA with DPPH$^{\bullet}$ (6.5 kJ mol^{-1}) and lower than for the reaction of *trans*-δ-viniferin with DPPH$^{\bullet}$ (31.9 kJ mol^{-1}) confirming that it is more favoured.

The SET mechanism is the least likely one for AA, compared to SPLET mechanism which is more preferred ($\Delta_r G^0$ =103.5 kJ mol^{-1}). On the other hand, the SET mechanism is more feasible than SPLET for tRes and *trans*-δ-viniferin, with $\Delta_r G^0$ values twice lower than those for SPLET.

Table 2. B3LYP/6-311++G(d,p) theoretical $\Delta_r G^0$ values for the reactions of DPPH$^{\bullet}$ with AA, tRes and *trans*-δ-viniferin in ethanol at 298.15 K, in kJ mol^{-1}. For the SET-PT and SPLET mechanisms, only the first stage was considered, as the rate limiting steps.

Compound	$\Delta_r G^0$ (HAT)	$\Delta_r G^0$ (SET)	$\Delta_r G^0$ (PL)
AA	6.5	137.0	103.5
tRes	2.4	55.9	116.6
trans-δ-viniferin	31.9	51.5	111.6

Therefore, the calculation results are in contradiction with the first research hypothesis, because on the basis of $\Delta_r G^0$ values, the reaction of *trans*-δ-viniferin with DPPH$^{\bullet}$ is not favoured as compared to the reaction between DPPH$^{\bullet}$ and tRes.

The second research hypothesis states that for AA, when added to a reacting mixture of tRes+DPPH$^{\bullet}$, it would be thermodynamically more feasible to react with the radical forms of tRes and/or *trans*-δ-viniferin and regenerate the species. The regenerated tRes and/or *trans*-δ-viniferin would then be again included into the process of DPPH$^{\bullet}$ quenching, thus increasing the overall antioxidant activity of the mixture.

To verify the above statements, the half-reactions for AA and the three different radicals (tRes$^{\bullet}$, DPPH$^{\bullet}$ and the radical form of *trans*-δ-viniferin) have been taken for further consideration, according to the following equations, similar to the previously mentioned ones:

HAT: $AA + Rad^{\bullet} \rightarrow AA^{\bullet} + Rad{-}H$ (15)

SET: $AA + Rad^{\bullet} \rightarrow AA^{\bullet+} + Rad^{-}$ (16)

PL: $AA + Rad^{\bullet} \rightarrow AA^{-} + Rad\text{-}H^{\bullet+}$ (17)

where Rad denotes tRes, DPPH or *trans*-δ-viniferin, respectively.

The values of the Gibbs Free Energy of the reactions ($\Delta_r G^0$) have been calculated according to the formulas similar to eq. 9–11:

$$\Delta_r G^0\,(\text{HAT}) = [G(AA^{\bullet}) + G(Rad{-}H)] - [G(AA) + G(Rad^{\bullet})] \quad (18)$$

$$\Delta_r G^0\,(\text{SET}) = [G(AA^{\bullet+}) + G(Rad^{-})] - [G(Rad^{\bullet}) + G(AA)] \quad (19)$$

$$\Delta_r G^0\,(\text{PL}) = [G(AA^{-}) + G(Rad\text{-}H^{\bullet+})] - [G(AA) + G(Rad^{\bullet})] \quad (20)$$

Calculation results (Table 3) show that only one reaction is characterized by an exergonic value of the Gibbs Free Energy, and that is the reaction between AA and the *trans*-δ-viniferin radical (–61.6 kJ mol^{-1}). The HAT mechanism is the most probable one for the reaction of AA with

all the three compounds, because it has the lowest values of $\Delta_r G^0$. It is also clear that the value of the Gibbs Free Energy of the reaction of AA with tRes• and the *trans*-*δ*-viniferin radical is lower than for the reaction of AA with DPPH•, confirming the second research hypothesis.

Table 3. B3LYP/6-311++G(d,p) theoretical $\Delta_r G^0$ values for the reactions of AA with tRes•, DPPH• and the radical of *trans*-*δ*-viniferin, in ethanol at 298.15 K, in kJ mol^{-1}. For the SET-PT and SPLET mechanisms, only the first stage was considered.

Compound	$\Delta_r G^0$ (HAT)	$\Delta_r G^0$ (SET)	$\Delta_r G^0$ (PL)
tRes•	4.1	173.8	84.6
DPPH•	6.5	137.0	103.5
trans-*δ*-viniferin	–61.6	147.3	11.5

The SET mechanism is the least likely one, given the highest value of the Gibbs Free Energy of the reaction, which is also verified by the highest values of IP, which characterizes the first step of the SET-PT mechanism.

The SPLET mechanism, which was shown to be the most probable according to the values of PA (Table 1), is characterized by higher values of the Gibbs Free Energy of the reaction than HAT, making it less feasible. However, it is interesting to observe than even in this case, the reactions of AA with the *trans*-*δ*-viniferin radical and with tRes• are thermodynamically more favoured, given the difference of around 90 kJ mol^{-1} and 20 kJ mol^{-1} in $\Delta_r G^0$, in comparison to the reaction of AA with DPPH•, respectively.

4. Conclusions

The antioxidant interactions between tRes and AA in scavenging the DPPH• were investigated by UV-Vis and ^{13}C NMR spectroscopies, showing different resultant antioxidant effect, *i.e.* antagonistic and synergistic, in dependence of the reaction conditions.

The UV-Vis experiments have demonstrated that the type of AI depended on the modalities of radical's interaction with the studied antioxidants. Thus, initial interaction of tRes with DPPH• followed by the addition of AA offered the strongest synergistic effect of 1.19, whilst simultaneous interaction of both antioxidants with DPPH• or the case when DPPH• firstly interacted with AA succeeded by the addition of tRes, showed strong antagonistic effects of 0.87 and 0.86, respectively. The tRes/AA molar ratio showed to be significant for the amplitude of the synergistic effect, the 1:1 tRes/AA molar ratio demonstrating the strongest synergy.

The ^{13}C NMR spectra have complemented the literature data about oxidative conversion of tRes into its dimer-*trans*-*δ*-viniferin upon interaction with DPPH•. On account of the NMR data, generation of *trans*-*δ*-viniferin has been found essential for manifestation of antioxidant's synergism for the portrayed system of antioxidants. The interplay between tRes and AA upon scavenging the DPPH• displayed by UV-Vis and ^{13}C NMR spectroscopies, may be examined as resembling natural processes substantiating the known information.

Computational studies showed that *trans*-*δ*-viniferin do not have a higher antioxidant activity towards DPPH• than tRes or AA. Therefore, given the slow kinetics of the tRes - DPPH•, it is not the depletion of *trans*-*δ*-viniferin that drives the reaction equilibrium, but it is rather the reaction of tRes and DPPH• that pushes the equilibrium towards the formation of *trans*-*δ*-viniferin. Once formed, this latter species competes with tRes for quenching DPPH•. Computational studies showed that AA regenerates both tRes• and *trans*-*δ*-viniferin radicals by the HAT pathway that requires less energy than the reaction of AA with DPPH•, and therefore the synergism can be, theoretically, attributed to this regeneration. The regenerated tRes and *trans*-*δ*-viniferin would then be again included into the process of DPPH• quenching, thus increasing the overall antioxidant activity of the mixture. More detailed computational research will follow in order to study the transition states and activation energies of the above discussed reactions.

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Povzetek

Povzetek: V tej študiji je bila uporabljena kombinacija UV-Vis, ^{13}C NMR spektroskopskih in računalniških metod za raziskovanje antioksidativnih interakcij med *trans*-resveratrolom in *L*-askorbinsko kislino v reakciji z radikalom 2,2-difenil-1-pikrilhidrazilom. Opaženi so bili pomembni sinergijski in antagonistični učinki, odvisno od pogojev reakcije. Preizkušena so bila različna molarna razmerja antioksidantov, pri čemer je bila najvišja sinergijska interakcija zabeležena pri razmerju 1:1. ^{13}C NMR spektri nakazujejo na dimerizacijo resveratrola, pri čemer nastane znana naravna spojina *trans*-δ-viniferin, kar predstavlja ključen pojav, vključen v sinergijsko antioksidativno delovanje. Podatki iz teorije funkcionala gostote (DFT) dopolnjujejo raziskavo in nakazujejo možnost sinergijskih interakcij preko regeneracije radikalov resveratrola in njegovih oligomerov z askorbinsko kislino po mehanizmu prenosa vodikovega atoma.