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Original Article

Gallic Acid Radical Generation in Aromatic Plants: A Combined EPR and UV-Vis Spectroscopic Approach

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ABSTRACT

Objectives: In the present work, five aromatic plants: *Origanum vulgare* L., *Rosmarinus officinalis* L., *Ocimum basilicum* L., *Salvia officinalis* L. and *Achillea millefolium* L., are examined in order to determine Gallic Acid (GA) radical generation in them by UV-Vis and EPR spectroscopy. **Methods:** The phenol content of plant extracts was estimated at 280 nm by deconvolution of UV-Vis spectra using a GA calibration curve whilst the radical activity was quantitated by EPR Spectroscopy at 77 Kelvin using the stable free radical DPPH• as a reference. **Results:** The radical activity ranged from 7×10^{12} spins in *O. vulgare* L. to 2.2×10^{13} spins in *O. basilicum* L. of DPPH•/g sample whilst the phenol content as GA ranged from 28.1 mg of GA/g sample in *O. vulgare* L. to 65.2 mg of GA/g sample in *A. millefolium* L. Moreover, EPR spectra showed that all samples contain stable radical signals with g-values 2.0046–49 and a line width of 3–5 Gauss. These are characteristic for π-type semiquinone radicals of GA compounds with the unpaired electron partially on the oxygen atom of the phenolic ring radicals. **Conclusion:** These significant differences between the UV-Vis and EPR measurements reveal that the stabilization of the radical fraction, type GA• in aromatic plants is a combined result of aromatic species and local effects; namely, phenolic groups and aromatic environment.

Key words: Antioxidant activity, Redox-active polyphenols, Deconvolution analysis.

INTRODUCTION

Aromatic plants are used in making perfumes, in cooking, in the food, pharmaceutical and liquor industries, while their use dates back to BC in the Middle East.[1] Nowadays, the United States Food and Drug Administration has recognized more than 150 aromatic plants that are safe for human consumption without limitations on intake.[2] Most of them belong to the *Lamiaceae* family, such as *Origanum vulgare* L. (*O. vulgare* L.), *Rosmarinus officinalis* L. (*R. officinalis* L.), *Ocimum basilicum L.* (*O. basilicum* L.*)*, *Salvia officinalis* L. (*S. officinalis*) and present strong antioxidant activity with redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, chelators, scavengers of free-radicals such as superoxide anion.[3-5] Aromatic plants are complex regarding their antioxidant activity on phenolic compounds.[6] Today, around 8,000 polyphenols have been reported as phenolic compounds with antioxidant activity in aromatic plants. These compounds can be divided into several subgroups that range from simple phenolic molecules, such as hydroxylated derivatives of benzoic acid [gallic acid (GA), protocatechuic acid, caffeic acid, rosmarinic acid, phenyl glycoside, 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxyphenyl], propionic acid, carvacrol and thymol] to polymerized phenolic compounds (polyphenols) such as tannins and flavonoids.[7-12] In case of the polyphenols, the structural arrangements imparting greatest antioxidant activity as determined from relative study are: the ortho 3′,4′-dihydroxy moiety (type gallol) in the B ring (e.g., in catechin, luteolin and quercetin), the meta 5,7-dihydroxy arrangements in the A ring (e.g., in kaempferol, apigenin and chrysin), the 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization (e.g., in quercetin), as long as the o-dihydroxy structure in the B ring (type gallol) is also present.[13] According to Giannakopoulos *et al.* polyphenols type GA contain the highest radical scavenging activity.[14-16] However, alterations in the arrangement of the hydroxyl groups, substitution of contributing hydroxyl groups by glycosylation and conjugation of the benzene groups can lead to decreases of antioxidant activity.[14,17,18] In addition, many factors can affect their phenolic content and antioxidant activity (as radical activity) such as: the chemical structure of analytes studied, the selected methods, the composition/nature of the aromatic plant and storage conditions.[6,19,20] In particular, the extraction yield is dependent upon the solvent polarity and extraction methodwhere the antioxidant capacity of aqueous herb extracts are monitored with different methods (Fe^{3+} reduction, DPPH, hydroxyl radical, Low-Den-

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sity Lipoprotein oxidation).[19,20] The earlier results revealed that at pH > 7 GA and its analogues are rapidly oxidized by atmospheric oxygen.[14,21] Hence, given the importance of free radicals (as antioxidant activity) in aromatic plants, the determination of total free radicals in the solution is difficult. Today, owing to its stable nitrogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2×10^{13} spins/mm^3 is commonly utilized in analytical chemistry to evaluate antioxidant activity by UV-Vis, Electron Paramagnetic Resonance (EPR) Spectroscopy, or other analytical techniques.[22-24]

In conclusion, the EPR technique coupled with UV-Vis spectroscopy can regarded as a good combination for determination of radical activity and total phenolic content in aromatic plants. In the present study, five aromatic plants *O. vulgare* L., *R. officinalis* L., *O. basilicum* L., *S. officinalis* L. and *Achillea millefolium* L. (*A. millefolium* L.*)* of the *Lamiaceae* and *Asteraceae* families, are examined in order to determine GA species generation and speciation in phenolic substances of aromatic plants by EPR and UV-Vis spectroscopy.

MATERIALS AND METHODS

Plant materials

Fresh aromatic plants *O. vulgare* L., *R. officinalis* L., *O. basilicum* L., *S. officinalis* L. and *A. millefolium* L., organically grown in Western Greece, were harvested in May 2019 (sup.doc). The experiment was carried out from February until May 2019 in a heated glasshouse located at the Technological Institute of Mesologhi in Greece as described earlier.[25] The aromatic plants were grown under natural light conditions. The air temperature inside the glasshouse was maintained between 18°C and 31°C during the day and 15°C to 21°C during the night. All samples were collected during flowering stage before fruiting. The leaves of the aromatic plants were cut and dried at 40°C in darkness for 5 days as described earlier.[25] Then, they were packed in paper bags under N_2 and stored for up to 2 months. The dry matter of harvest was pooled and mixed for the chemical analysis. All samples were analyzed within one month of collection.

Determination of GA• radicals by EPR spectroscopy

Total antioxidant activities of dried aromatic plants were determined by EPR spectroscopy. Continuous wave electron paramagnetic resonance spectra were recorded at cryogenic temperatures 77 Kelvin with either a Radiopan SE or a Bruker Bruker ER200D spectrometer operating at X-band frequencies. The Bruker spectrometer with a 100 kHz magnetic field modulation was equipped with Bruker NMR gaussmeter ER 035M and Agilent microwave frequency counter 5310A. *g*-values were calibrated versus DPPH, *g*=2.0036, which was also used as spin standard as described earlier.[14,26-28] Also, the quantitative EPR technique was applied (microwave power, 2 mW; modulation amplitude, 1G; 20.0 mg sample; standard quartz tubes; etc.). Simulation of the EPR spectra was performed by using the program WINEPR-SimFonia, version 1.25 by Bruker. All determinations were performed in triplicate.

Extraction and quantitation of GA species by UV/Vis spectroscopy

The extraction method used for dried samples was as follows: 100 ml of a mixture of Methanol, Formic acid and Milli Q water at a volume ratio of 50:1.5:48.5 (extraction Solution M:F:M) were added to 1 g of dried sample. The mixture was stirred carefully for 60 min. The mixture was then filtered and centrifuged at 10.000 *g* (type: Centra-MP4R of IEC, USA) for 10 min at 4°C.[29] The supernatant from centrifugation was filtered through a 0.45-μm filter and then was used for the determination of total phenolics via a UV spectrophotometer Shimadzu Corp., Japan UV-1601. Standard solution, for determination of total phenolics was prepared by dissolving 10 mg GA, in 100 ml M:F:W solution to obtain the concentration of 0.59 mM (stock solution). The results were expressed as milligrams of GA equivalents per gram of dry weight and are presented as means of triplicate analyses. The absorbance relative to that of blank prepared using M:F:M extraction solution was measured at 280 nm by deconvolution of UV-Vis spectra and quantitated using a GA calibration curve. According to Figure 3 (insert), the UV-Vis spectrum for GA standard in M:F:M solution extends from 260 to 320 nm with a maximum absorbance at wavelength around 282 nm.

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation. The significance of differences between treatment means was evaluated by applying one-way analysis of variance ($p \leq 0.05$) using the origin software package.

RESULTS

GA• radicals determination in aromatic plants by EPR spectroscopy

Figure 1 shows that the EPR spectra are strong and generally similar for all the aromatic plants studied and depict monomeric stable radicals.[14]

In particular, all the EPR spectra are confined in a narrow magnetic field area ranging between 3,375 and 3,410 Gauus with a *g*-value of 2.0046-49 ± 0.0002 and a line width of 3–5 Gauss. The quantitative estimation of GA• radicals, by double integrating the EPR signal, yields the highest radical concentration of 2.2×10^{13} spin of DPPH•/mm^3 in the *O. vulgare* L., while the *O. basilicum* L. yields the lowest radical concentration of 7×10^{12} spin of DPPH•/mm^3 in all the examined aromatic plants. Based on Figure 1, the antioxidant activity of aromatic plants are arranged in descending order from the highest to the lowest as follows: *Origanu* > *Salvia* > *Achillea* > *Rosmarinus* > *Ocimum*, with radical concentration 2.2×10^{13}, 1.7×10^{13}, 1.5×10^{13}, 9×10^{12} and 7×10^{12} spins of DPPH•/mm^3, respectively (Figure 2).

Spectrophotometric quantification of GA in aromatic plants by deconvolution

The UV/Vis spectrum of the *R. officinalis* L. (Rosemary) plant extract is shown in Figure 3.

Figure 3 shows two main peaks at wavelengths around 280 and 330 nm, while similar UV-Vis spectra present the other aromatic plants. At this point, deconvolution analysis is applied in order to determinate the behavior and magnitude of two (or more) components contributing to the UV/Vis spectum. The results of UV-Vis deconvolution-fit spectra for all aromatic plants are summarized in Table 1. As reported by Stintzing *et al.* these two bands (Figure 3) originate from π–π^* transitions of electron.[30]

Based on deconvolution analysis of UV/Vis spectra (Table 1), the first wavelength (1st Gaussian component) for maximum absorbance by every aromatic plant is found in a narrow wavelength band of approximately 280 nm (272.1 to 285.4 nm). The second wavelength (2nd Gaussian component) for maximum absorbance is found out in a wide wavelength band at longer wavelengths ranging from 310.1 to 375.7 nm. A comparison of UV/Vis GA spectrum with measured spectra for all plant extracts reveals that the first absorbance band (at 280 nm) displays optical properties that are characteristic of GA phenolic compounds (Figure 1, inset).[14,15] In this context, the GA content of aromatic plants was calibrated against a GA stock solution as described earlier and is expressed

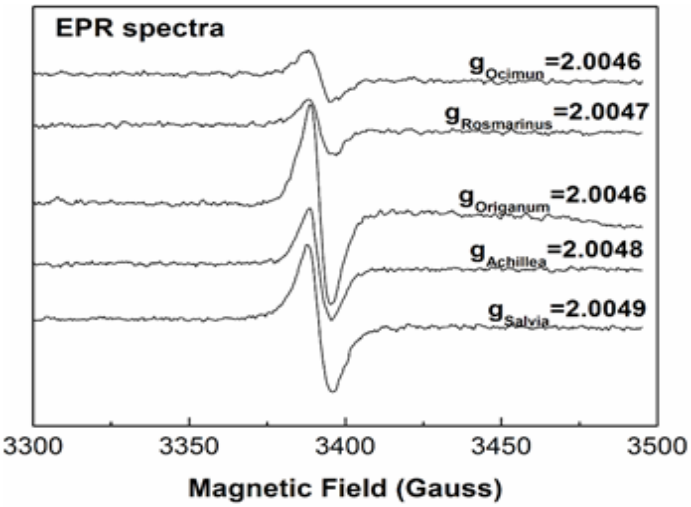

Figure 1: EPR spectra at 77 oK of *O. vulgare* L., *R. officinalis* L., *O. basilicum* L., *S. officinalis* L. and *A. millefolium* L. The specrta were performed as means of triplicate analyses.

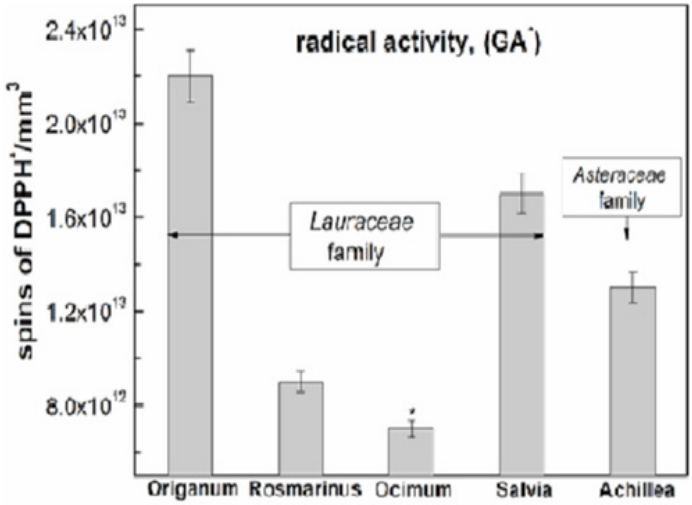

Figure 2: Radical activity, GA●, expressed as spins of DPPH●/1 mm3 of *O. vulgare* L., *R. officinalis* L., *O. basilicum* L., *S. officinalis* L. and *A. millefolium* L. The results were determined as means of triplicate analysis. Error bars show standard deviations from three repeated experiments. Significant differences between means are indicated by asterisks (*) at $p \leq 0.05$.

as mg equivalent of GA/g dry weight of aromatic plants in Figure 4. The reproducibility of the method was determined by analyzing the total phenolic content in five samples with a Relative Standard deviation of 3.5% which demonstrates very good repetition.

According to Figure 4, the *O. vulgare* L. has a GA value approximating 65.2 mg of GA/gr dry weight. This value is the highest amongst all the aromatic plants, whereas the *A. millefolium* L. has the lowest GA value approximating 28.1 mg of GA/gr dried weight. Moreover, the phenolic content of five aromatic plants, expressed as mg of GA/gr dry weight, are arranged in a descending order from highest to lowest as follows: *Origanum* > *Rosmarinus* > *Ocimum* > *Salvia* > *Achillea* with GA values of 65.2, 62.1, 50.0, 48.2 and 28.1 mg of GA/gr dry weight, respectively. We furthermore noticed that aromatic plants of the *Lamiaceae* family had a higher GA values than the *Achillea* of the *Asteraceae* family. This result is confirmed by other relative studies, where the aromatic plants of the *Lamiaceae* family exhibit a strong antioxidant activity[4] and include many phenolic compounds which act as powerful antioxidants and free radical

Table 1: Half-wavelength values ($w_{1/2}$) used for the Gaussian deconvolution of the UV-Vis spectra.

Aromatic plants	$w_{1/2}$ (nm) (±0.1 nm) Gaussian components		
	1	2	R2
O. vulgare L.	272.1	310.1	0.99897
R. officinalis L.	274.9	334.8	0.99985
O. basilicum L.*	275.2	375.7	0.99889
S. officinalis L.	285.4	350.1	0.99982
A. millefolium L.*	278.6	365.3	0.99981

*Significant differences between means are indicated by asterisks at $p \leq 0.05$.

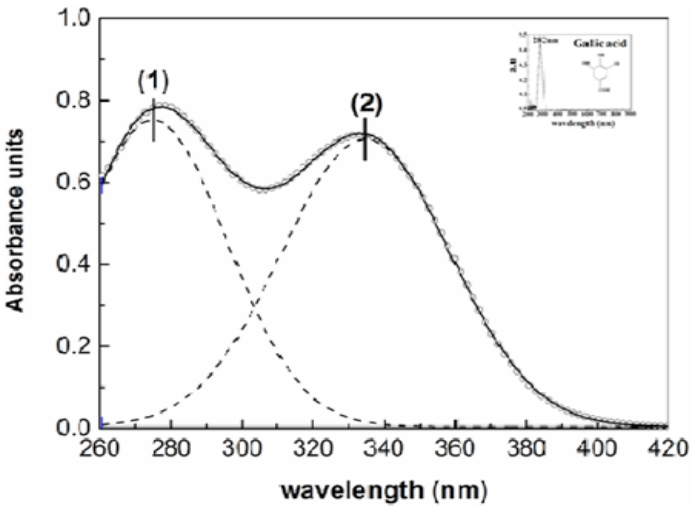

Figure 3: Deconvolution fit of UV-Vis spectrum for *R. officinalis* L. plant extract, (circles) experimental data, (dashed and dotted line) Gaussian components (solid line), sum of theoretical peaks. The thick bars indicate the wavelength for maximum absorbance values (at half-wavelength w1/2) used for the fit, listed in Table 1. Insert shows the UV-Vis spectrum of GA in the extract solution.

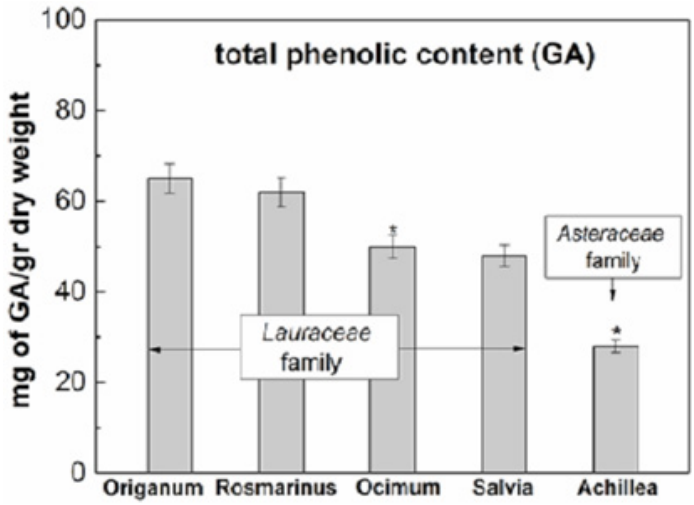

Figure 4: The phenol content of *O. vulgare* L., *R. officinalis* L., *O. basilicum* L., *S. officinalis* L. and *A. millefolium* L. plants extracts, expressed as mg of GA/gr dry weight. The results were determined as means of triplicate analysis. Error bars show standard deviations from three repeated experiments. Significant differences between means are indicated by asterisks (*) at $p \leq 0.05$.

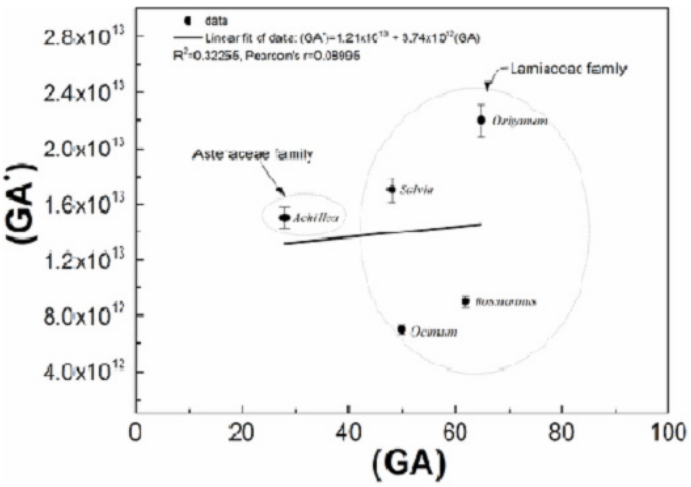

Figure 5: Correlation between radical activity, GA● and total phenol content expressed as GA of examined aromatic plants (R^2 = 0.32255, Pearson's coefficient, r = 0.08995).

scavengers.[31,32] According to Chrpová *et al*. it is difficult to compare the total phenolic content values that are published in many papers, since the data is significantly influenced by the extraction method and analytical method used in their determination; so in some cases, the results vary in accordance with the analytical method.[33] The results obtained in this study are in relatively good agreement with the data in the literature, where the authors determined the total phenolic content.[3,5,25,33,34]

DISCUSSION

Free radical scavenging profile

As previously mentioned, several studies have shown that these EPR signals (Figure 1) are characteristic of π-type semiquinone radicals of GA and bear a strong resemblance to the indigenous radicals of natural.[14,35] According to Kiokias *et al*. this GA radical activity is due to donate hydrogen atoms from GA to others free radicals-R• (reaction 1).[36] This oxidation of phenolic compounds at near-neutral pH produce semi-quinone free radicals, GA• that is easily observed by EPR spectroscopy.[14,37]

In addition, Shahidi and Ambigaipalan reported that phenolic hydroxyl radicals, GA• are stabilized by the delocalization of their unpaired electron around the aromatic ring.[38]

Moreover, according to Jurd (1957) the second wavelength (2nd Gaussian component) displays optical properties that are characteristic of quercetin (flavonol class) due to conjugated benzene groups according to following mechanism,[18] (reaction 2, 3):

As reported by Jurd and Giannakopoulos *et al*. The above formation of two distinct polymers by free radical mechanism (reactions 2, 3) signify a very low redox radical activity of the phenolic content.[16,18,39] These mechanisms demonstrate the presence of different amounts of GA and various degrees of redox radical activity (Figures 2 and 4) in aromatic plants.

Quantitative correlation between radical activity GA• and GA compounds

The correlation between GA and GA• species was investigated for all aromatic plants (Figure 5). According to Figure 5, there is not a high correlation between GA• and GA content of aromatic plants. In particular, the statistic results at $p \leq 0.05$ level (Figure 5) indicate a low R-square value ($R^2 = 0.32255 < 1$) and very low Pearson's r correlation coefficient value, $r = 0.08995 << 1$.

Furthermore, the slope is not significantly different to the zero and bivariate correlation between the two variables is very low. Moreover, according to Figure 5, the results for case of the aromatic plants of *Lamiaceae* family show that our model doesn't fit on data o very well and there is consistently not a very good correlation between GA and GA•. These significant differences between the UV-Vis and EPR measurements for GA speciation reveal that a fraction of the GA content corresponds to redox radical activity, GA• in the aromatic plants. According to other studies, this fraction is strongly dependent upon local effects such as p-stacking, where hydrophobic sequestration modulates the stability of the hydroxyl phenol radicals in natural polyphenol matrices such as HS.[14,16] Thus, the stabilization of the GA• radicals in aromatic plants is a combined result of local effects, namely, phenolic groups, redox and aromatic environment.[14,16,38]

CONCLUSION

The results demonstrate the presence of different species of GA and various degrees of redox radical activity that is dependent upon the type of aromatic plant species (Figures 2 and 4). Moreover, EPR spectra demonstrate that all the samples contained a stable radical signal with g-values of 2.0046–49 and a line width of 3–5 Gauss. These are characteristic of the π-type semiquinone radicals GA• of GA, where the unpaired electron is partially on the oxygen atom of the phenolic ring. Also, the significant differences of the GA concentrations between the UV-Vis and EPR measurements reveal that a fraction of the total phenolic accumulation, type GA corresponds to redox active antioxidant charge in aromatic plants and that the stabilization of the GA• radicals in aromatic plants is a combined result of aromatic species and local effects; namely, phenolic groups and aromatic environment. In light of the above results, GA can adopted as a good model evaluation of the redox antioxidant activity of aromatic plants.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

GA: Gallic acid; **GA•:** Gallic acid free radical; **EPR:** Electron Paramagnetic Resonance; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **DPPH•:** 2,2-diphenyl-1-picrylhydrazyl free radical; **HS:** Humic substances.

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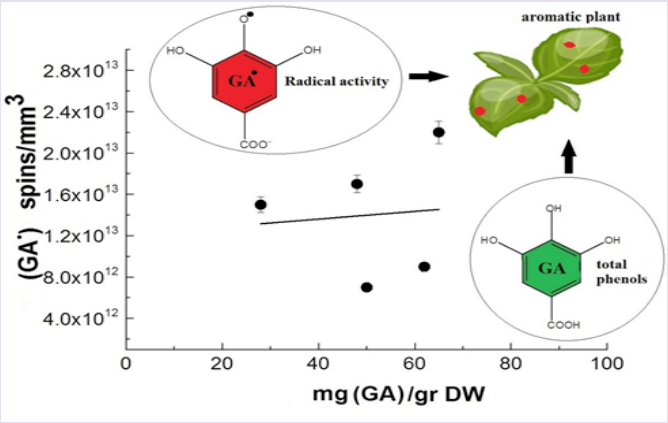
GRAPHICAL ABSTRACT



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SUMMARY

- Polyhydroxy phenolic compounds (Ph-OH):
- (i) are found widely distributed in various plants
- (ii) have a diverse range of industrial uses, as antioxidants in food, in cosmetics, and in the pharmaceutical industry.
- (iii) donate a proton from their hydroxyl (O-H) bond through hemolytic cleavage and form a stable phenoxy radical, type GA●.
- The Combined EPR and UV-Vis spectroscopic study reveals that a fraction of Ph-OH corresponds to redox antioxidant activity in aromatic plants.
- EPR/UV-Vis results show that the GA● can adopted as a good model of phenoxy radicals in aromatic plants.

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