Letter

# Leaves are a Source of Biogenic Persistent Free Radicals

Eric P. Vejerano* and Jeonghyeon Ahn

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**ABSTRACT:** Nonsenescent and senescent leaves of selected coniferous and broadleaf plants contained substantial levels of naturally occurring persistent free radicals (PFRs). These biogenic PFRs (BPFRs) were stable and persistent despite multiple wetting and drying cycles, implying that BPFRs can leach and sorb on soil particles. Results suggest that endogenous chemicals in plants and their transformation byproducts can stabilize unpaired electrons in leaves under ambient conditions. Thus, the vast amount and perpetual supply of leaf litter is an unaccounted natural source of BPFRs. If toxic, inhaling and accidentally ingesting fine soil dust and powder from degraded leaf litter may increase our environmental and health burdens to PFRs. We expect that this finding will generate more studies on natural sources of PFRs, establish their properties, and distinguish them from those formed from combustion and thermal processes.

**KEYWORDS:** *organics, EPFRs, aging, stress, plants*
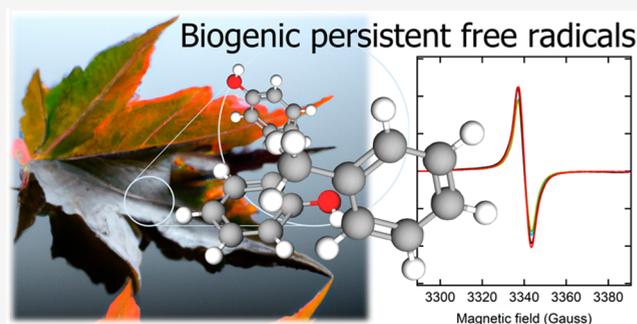
## INTRODUCTION

Environmentally persistent free radicals (EPFRs), first reported in charcoal by Ingram and colleagues[1] in 1954, are stable and exhibit long lifetimes.[2] Over a decade of research on EPFRs has established their adverse health effects.[3] EPFRs exist in various environmental matrices,[4−6] but research efforts have focused mainly on sources originating from combustion and thermal processes. The surface-assisted model[2] is the most invoked of the three models of EPFR formation.[7] In this model, EPFRs are posited to form when an aromatic compound reacts on the surface of a transition metal oxide that proceeds via three key steps: physisorption, chemisorption, and electron transfer.[2,8] This conceptual model assumes that a transition metal oxide surface is critical to stabilizing the unpaired electron,[2] without which EPFRs cannot form. However, such a condition is not necessary. Two studies have shown that some oxygenated and nonoxygenated polycyclic aromatic hydrocarbons react with ozone and hydroxyl radical ($^{\bullet}OH$), forming EPFRs without a transition metal oxide surface.[9,10]

Thus, we wonder if organic compounds in plants or metabolic byproducts of multiple processes during a plant's natural development and aging (e.g., senescence and stress) can stabilize unpaired electrons to form PFRs. We hypothesize that plant leaves generate and contain substantial stable and persistent organic free radicals since electron donor−acceptor processes (e.g., photosynthesis[11] and respiration[12]) abound in leaves. If so, leaves are an unaccounted source of PFRs.

In the extant literature, we found reports of long-lived organic free radicals in other plant structures formed at certain conditions: in the woody tissue of a sycamore tree when infected by a fungus,[13] in coffee beans when roasted,[14,15] in seeds and pollen during artificial aging[16] and dehydration,[17] and in wheat leaf tissue when exposed to high ozone levels.[18] Stable organic free radicals in fully senescent broadleaves are mentioned briefly in one study.[19] To the best of our knowledge, there are no studies of stable organic free radicals in nonsenescent broadleaves, in both senescent and non-senescent broadleaves, and in coniferous leaves, particularly if these radicals are stable and persist indefinitely like EPFRs.

## MATERIALS AND METHODS

**Leaf Samples.** To test our hypothesis, we collected nonsenescent and senescent leaves without visible signs of pathogen inclusion, at least to the naked eye, and that were still attached to coniferous trees loblolly pine (*Pinus taeda*), thuja (*Thuja plicata x standishii*), and cedar (*Cedar spp*) and broadleaf crape myrtle (*Lagerstroemia speciosa*), birch (*Betula papyriferia*), and red maple (*Acer rubrum*) trees in the wild in Fall 2021 and Spring 2022. The nonsenescent and senescent leaf samples were collected from the same tree. Also, in Spring 2023, select birch and crape myrtle branches were covered
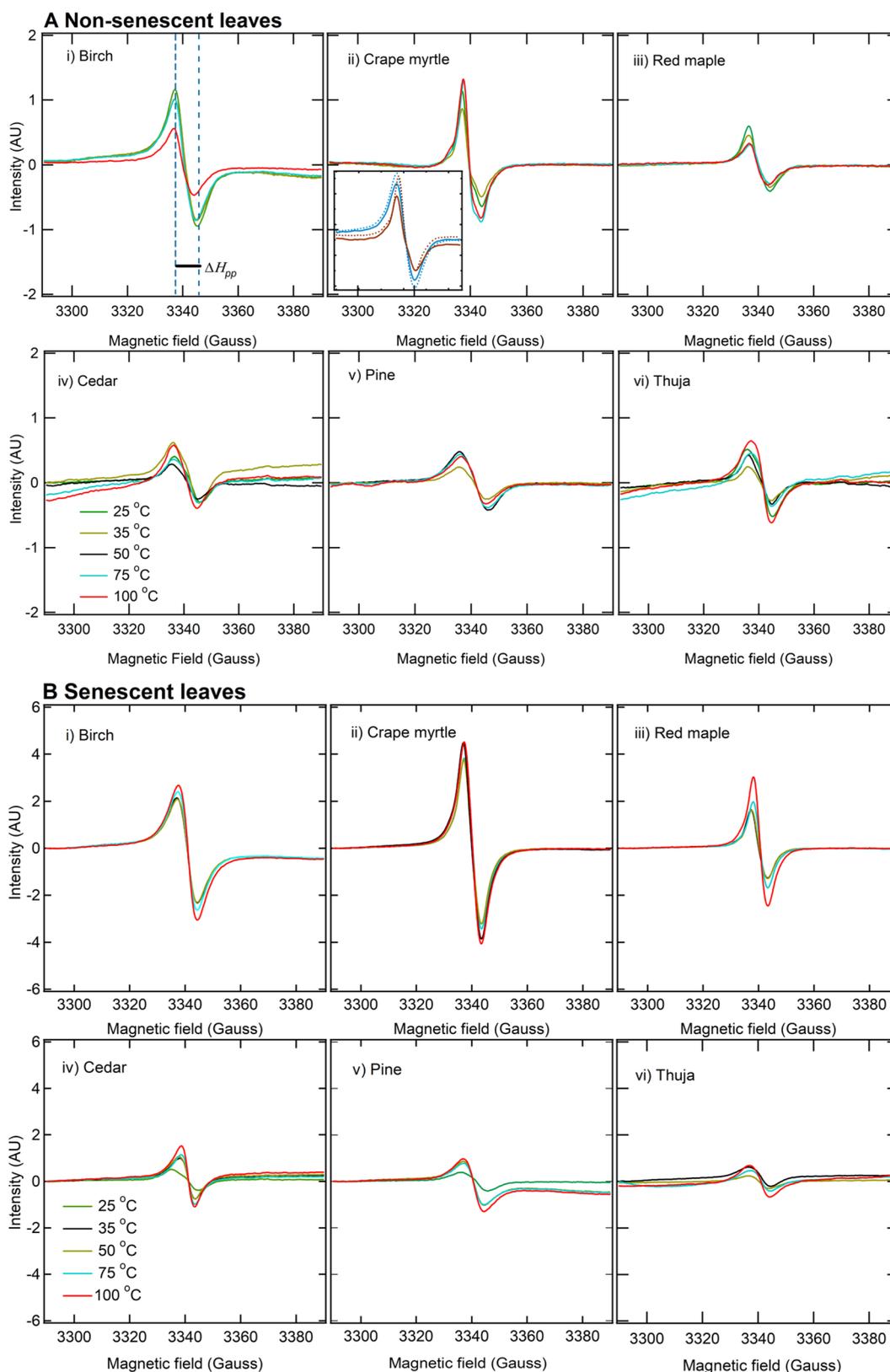
**Figure 1.** First-derivative ESR signals in (A) nonsenescent and (B) senescent leaves of broadleaf and coniferous plants. (A-ii, inset, plot is on a different scale) First-derivative ESR signals of covered (solid curves) and uncovered (dashed curves) nonsenescent birch (blue curves) and crape myrtle (brown curves) leaves. The masses of leaf samples were similar (100 ± 0.5 mg) for all samples (A-ii, inset).

with thin transparent polyethylene bags. We used these plastic bags to protect emerging leaves from airborne particulate matter (PM). We installed two plastic syringe tubes tied at the terminal and distal ends of overhanging branches. The tube contained loosely packed glass wool (2 in. thick) to ensure gas exchange and drain condensed water from leaf transpiration.
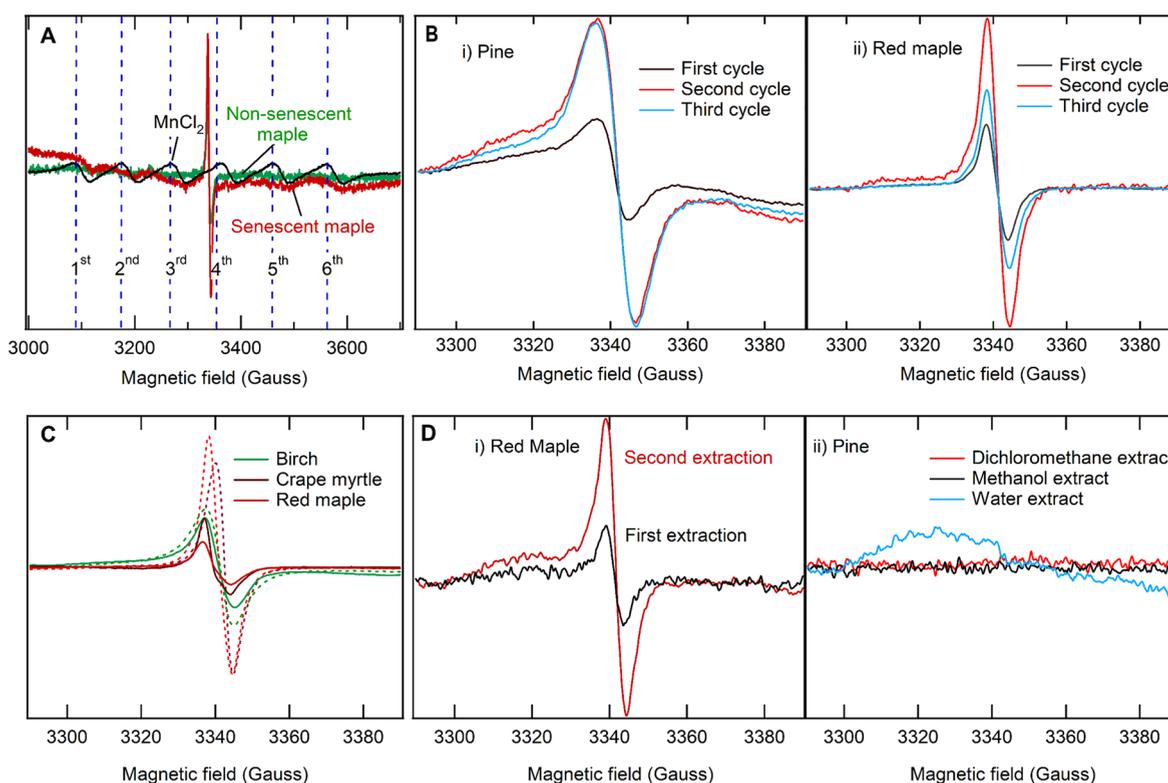
**Figure 2.** (A) First-derivative ESR spectra of BPFRs from nonsenescent and senescent red maple leaves scanned over a broad magnetic field showing the weak sextet $Mn^{2+}$ ESR signals (indicated by the blue dashed lines) in senescent leaves. The black curve is for $MnCl_2$, which was used for comparison. (B) ESR spectra of pine (i) and red maple (ii) after each cycle of wetting and drying. Plots are on the same scale. (C) ESR signals before (solid curves) and after (dashed curves) continuous cycles of wetting and drying (25 °C) over 6 days showing an increase in signals. (D) ESR signals from the water-soluble fraction of dried extracts of red maple (i) and pine (ii) extracted with different solvents. Plots are on the same scale.

We replaced the bags every week or when damaged. Four weeks following leaf emergence, we collected the leaves and their respective controls (uncovered leaves) from branches originating from the same primary branch. We removed dust from the uncovered leaves using a brush. Leaves were air- or oven-dried at 25 and 35 °C to reduce their moisture content so we could tune the electron spin resonance (ESR) spectrometer, but low enough to minimize altering the composition of the compounds in leaves. We dried resinous leaves at 35 °C for 72 h to remove moisture. Based on the ESR parameters for samples dried at 25 and 35 °C, drying at 35 °C did not alter the properties of the PFRs in leaves (Table S1).

Additionally, we used this temperature since the average summertime temperature can reach 35 °C in many places. After drying, we ground the leaves using a mortar and pestle. Ground leaves were sieved using 250-$\mu$m mesh. We used ~100 mg of leaves for ESR measurement. Note that we used the exact mass of leaf samples to calculate the concentration (spins per gram of leaves) in Table S1. Sieving and using similar quantities of samples ensure consistent packing densities on ESR tubes, resulting in a uniform distribution of the magnetic field and microwave within the ESR cavity resonator across various samples. Additionally, plant leaf samples were heated at 50, 75, and 100 °C to determine the effect of temperature on PFRs.

**Wetting and Drying Cycles.** Leaves of pine and red maple were subjected to three cycles of wetting (at their water-holding capacity) and drying to determine whether the PFRs were stable despite being continually subjected to natural wetting and drying cycles as in the case of leaves in the environment. For each cycle, we added water to wet the entire sample and then dried it at 35 °C for 12 h. We also performed parallel experiments for the senescent broadleaves, except that the bulk water added was evaporated at ambient conditions (22 °C), which took 2 days for each cycle, a total of 6 days.

**Extraction.** We extracted samples of red maple and pine leaves to determine whether the PFRs are soluble or deactivated by water. We extracted 500 mg of ground leaf samples using water (18 Ohm), methanol (Sigma-Aldrich), or dichloromethane (Sigma-Aldrich). The samples were vortexed for 5 min, then centrifuged for 10 min at $10{,}000 \times g$. The supernatant was pipetted into a Teflon filter and dried at 35 °C. The dried extract was ground before ESR measurement (detail in the Supporting Information).

**ESR Analysis.** We determined the g-factors from the peaks of the absorption spectra, which we obtained by converting the first-derivative ESR spectra in Figure 1 using Xenon software (Bruker). We corrected the g-factors using 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) as the reference standard (g-factor = 2.0036). We determined the line width ($\Delta H_{pp}$) as the difference in the magnetic field of the first-derivative ESR spectrum's minimum and maximum intensities (illustrated in Figure 1A-i).

## RESULTS AND DISCUSSION

**All Leaf Samples Contained Strong ESR Signals.** We detected substantial paramagnetic signals in the leaves of senescent and nonsenescent coniferous (cedar, pine, and

thuja) and broadleaf (birch, crape myrtle, and red maple) plants (Figure 1A and B). Broadleaf plants (Figure 1A-i, -ii, and -iii and 1B-i, -ii, and -iii) had higher ESR signals than the leaves of coniferous plants (Figures 1A-iv, -v, and -vi and 1B-iv, -v, and -vi). The [PFR] in broadleaf was higher than in coniferous leaves. Overall, the [PFR] in senescent leaves was ~2× higher than for the nonsenescent leaves (Table S1.) The corrected (see Materials and Methods) calculated concentration was ~$10^{15}$–$10^{16}$, which is 100× less than the [EPFR] in ambient $PM_{2.5}$. The [PFR] in leaves was orders of magnitude lower than in other matrices such as those in $PM_{2.5}$ and tobacco smoke (~$10^{16}$–$10^{18}$ spins/g),[4−6] biochar (~$10^{18}$ spins/g),[20,21] wildfire charcoal (~$10^{19}$–$10^{20}$ spins/g),[22] and $PM_{2.5}$ from extreme haze event (~$10^{20}$ spins/g).[23] Also, the [PFR] values from all samples stored as dried powder for 1 year remained nearly the same. The $\Delta H_{pp}$ and g-factors differed between plant species, indicating different PFRs.

**Are These ESR Signals Endogenous?** Leaves efficiently intercept airborne PM that contains EPFRs.[24] Leaves can internalize some fractions of fine and ultrafine PM via uptake through the cuticle or stomatal openings.[25] According to a previous study, pine leaves capture the most $PM_{2.5}$.[26] We assume that the pine leaves are cylindrical. On average, the width of the pine leaves we used is ~2 mm and a length of 80 mm, thus, a mean surface area of 2.5 cm². We used the value for pine leaves that captured 20 $\mu$g/cm² of $PM_{2.5}$.[26] Since we obtained the sample in Irmo, SC, which is 25 km from downtown Columbia, SC, we used the average [EPFR] in clean cities,[24,27] $10^{16}$ spins/g of $PM_{2.5}$ (similar to that in Rubidoux).[27] On average, the [$PM_{2.5}$][28] in Irmo, SC is <7 $\mu$g/m³, whereas those in Beijing were ~38 $\mu$g/m³.[26] Thus, 100 mg of leaf samples contained $10^{12}$ spins; this level represents an upper-bound estimate. The calculated concentration of $10^{13}$ spins/g of leaves is 2−3 orders of magnitude lower than those measured in the leaf samples, and thus, should be negligible.

To confirm if the PFRs are indeed endogenous, in Spring 2023, we measured the ESR signals from the leaves protected from airborne PM. If the ESR signals in leaves arise from EPFRs from intercepted and internalized airborne PM, we expect no ESR signals in the covered leaves. Instead, we detected strong ESR signals from the covered leaves (Figure 1A-ii, inset; a larger detailed image is depicted in Figure S1), confirming that the ESR signals are endogenous, and thus, biogenic. We attribute the slightly reduced ESR signals in the covered leaves (Figure 1A-ii, inset) to the reduced photosynthetic activity. The covered leaves had a paler green hue than those without the plastic covers. Water from leaf transpiration that condensed on the walls of the plastic cover attenuated the light transmission. Hereafter, we will call the PFRs in plant leaves biogenic PFRs (BPFRs) to distinguish them from EPFRs, which are stabilized on metal oxide surfaces and/or primarily formed via thermal/combustion processes.

**ESR Signals Originate from Organic Free Radicals.** The g-factors of the organic free radicals in leaves were 2.0031−2.0044, indicative of organic species. The organic free radicals coincide with the fourth peak of the weak, yet discernible, sextet $Mn^{2+}$ signal (Figure 2A, red curve) for senescent red maple leaves. It is plausible that plant components can stabilize the unpaired electron via steric and captodative effects, as observed for some synthetic organic free radicals.

**Potential Compounds That Are Responsible for These ESR Signals.** The BPFRs are unlikely to be chlorophyll

(Chl), such as photosystem II, chlorophyll z (Chl$_z$), $\beta$-carotene (Car), and cytochrome b559, despite being electron donors.[29] Our BPFRs differ from these radicals for two reasons. First, although the unresolved g-factor of the BPFRs (g > 2.0031) is close to the g tensor for the Car cation (Car$^+$) radical, the canthaxanthin cation radical, or the Chl$_z$ cation (Chl$_z^+$) radical,[29] the $\Delta H_{pp}$ values of the BPFRs at 25 or 35 °C (Table S1) were narrower than those for a Chl system ($\Delta H_{pp}$ = 9 G) signal of photosynthesis.[30] Second, the BPFRs were highly stable and persistent under ambient conditions, whereas Chl systems only generate transient ESR signals at −193 °C and under illumination.[29]

We speculate that the unpaired electron is stabilized by photosynthetic breakdown intermediates since the [BPFR] was higher in senescent leaves. Chl degradation involves multiple electron transfers,[31] a key step in free radical formation. Chl degradation massively occurs during leaf senescence,[32] but also in nonsenescent leaves, particularly when the photosynthetic activity of plants becomes overexcited,[32] i.e., under abundant light. Such a fact may explain our finding of the slightly higher [BPFR] in senescent leaves than in nonsenescent leaves and consistent with lower [BPFR] in the covered leaves. Efforts are underway to investigate the effect of the extent of photosynthetic activity on BPFR generation. Indeed, a study reported that the degradation of Chl systems in fully senescent plants coincided with the formation of unknown stable organic radicals.[19] Stable free radicals have been detected in the wood and stem of hardwood, softwood, and straw,[33,34] which is believed to result from lignin degradation and oxidation. However, the lignin in cell walls of senescent and nonsenescent leaves is unlikely to decompose while attached to the plants or during aging and stress. Organic compounds generated during or regulating senescence or other phytoactivities are potential candidates.

**BPFR Properties and Yield Change with Temperature.** For the senescent coniferous leaves, the $\Delta H_{pp}$ narrowed as the temperature increased, coupled with a slight shift in the g-factor, suggesting changes in the BPFR structures or transformation. The $\Delta H_{pp}$ and g-factor for the nonsenescent leaves varied more with increasing temperature than in the senescent leaves. Such shifts suggest that the radical structure changes with temperature despite being stable. The BPFRs in senescent leaves exhibited nearly constant $\Delta H_{pp}$ and g-factors except at higher temperatures, indicating being highly oxidized, and thus are resistant to further conversion to secondary free radical structures during heating. The ESR spectra for coniferous leaves formed a shoulder, suggesting that heating forms new PFRs or the original BPFRs are transformed into secondary free radicals.

**BPFRs are Highly Stable and Persistent Unaffected by Water and Drying.** High humidity can rapidly deactivate EPFRs,[35−37] especially in the natural environment where leaf litter is submerged in bulk water for an extended time. Surprisingly, we found that the BPFRs in pine (Figure 2B-i) and red maple (Figure 2B-ii), which we used as surrogates for coniferous and broadleaf plants, persisted despite being subjected to continuous wetting and drying cycles (Figure 2C). Overall, cycles of wetting and drying increased the [BPFR] (Figure 2B and C), suggesting impressive stability and persistence under this condition. With this stability in water, we determine if the BPFRs are extractable in water since leaching of BPFRs and sorption to soil particles ultimately will determine their environmental transport. Results indicate that

senescent red maple leaves had a high water-soluble fraction of BPFRs, which increased after the second extraction (Figure 2D-i). We did not detect ESR signals from the pine leaves (Figure 2D-ii) extracted with water, methanol, or dichloromethane. Overall, these results further support the fact that the organic molecular structures are different in pine and red maple as deemed from their water affinities.

**Potential Role and Function of BPFRs in Leaves.** The fact that stable free radicals are present in leaves regardless of the leaf type and senescence stage suggests that BPFRs serve essential functions at different life stages of plants. Indeed, there is a report that stable organic free radicals accumulate with an increase in superoxide anion radical ($O_2^{\bullet-}$) concentration in fully senescent leaves;[19] presumably, BPFRs may scavenge smaller and highly reactive free radicals (e.g., $O_2^{\bullet-}$ and $^{\bullet}OH$) during stress-related and age-dependent senescence. Moreover, the $Mn^{2+}$ peak was more pronounced in senescent leaves (Figure 2A, red curve), probably because $Mn^{2+}$ protects plants from oxidative stress[38] as their photosynthetic apparatus is being dismantled during autumnal senescence. Thus, we predict that BPFRs are ubiquitous in the leaves of higher plants because of their protective functions.

**Implications.** Perhaps, an important implication of this finding is that leaves are an unaccounted natural source of BPFRs since plants constitute ~82% of the Earth's land biomass.[39] Despite the orders of magnitude lower [BPFR] than [EPFR] from various matrices as described above, the amount of BPFRs can be substantial, because plants generate leaf litter continuously. Also, our finding suggests that a substantial amount of the PFRs in soils are BPFRs from leaves, which are difficult to discriminate from humic acids.[40]

BPFRs are contained in leaves and do not readily pose health risks, even if they exert similar adverse effects similar to EPFRs. However, the ultimate disintegration of leaf litter, the leaching of water-soluble BPFRs, and their adherence to soil particles can disperse them, thus creating potential exposure from them.

Overall, this preliminary research provides an exciting avenue for the origin and existence of PFRs from overlooked biogenic sources. Future studies should be devoted to (a) identifying the molecular structures of BPFRs, (b) investigating their toxicity, (c) understanding their fate and transport in the environment, for instance, long-term studies of their persistency over the lifetime of leaves as they are transformed to fine dust in the natural environment, (d) understanding their role in plants, and (e) investigating the mechanism by which BPFRs are formed.

## ■ ASSOCIATED CONTENT

### ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.3c00277.

Materials and methods for ESR measurement and quantification, confirmation of ESR signals being endogenous, shift in the ESR properties as a function of temperature (PDF)

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### Notes

The authors declare no competing financial interest.

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