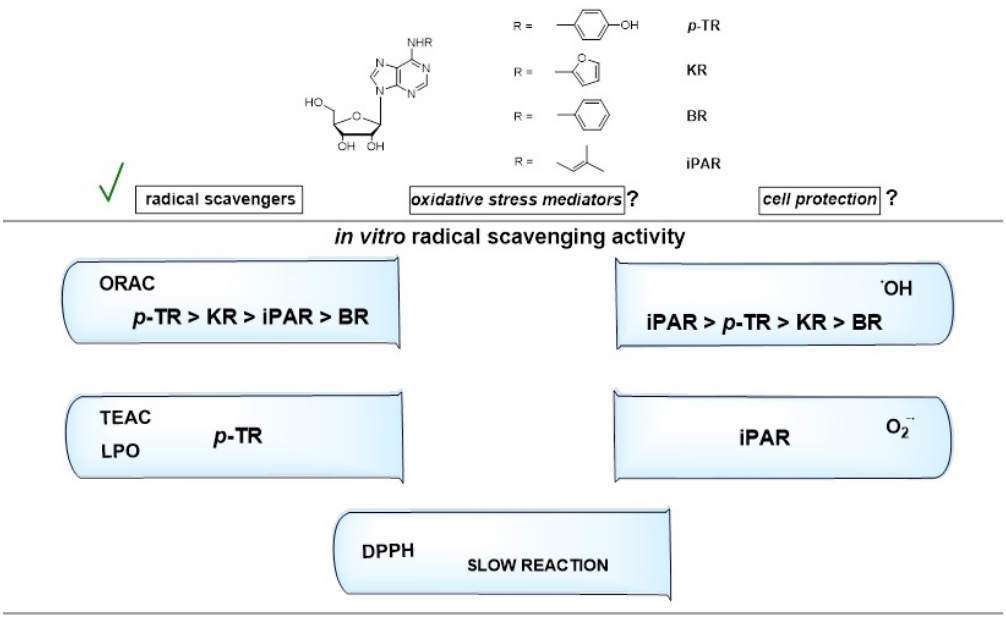

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Evaluation of the radical scavenging activity of some representative isoprenoid and aromatic cytokinin ribosides (N^6-substituted adenosines) by *in vitro* chemical assays

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Cytokinins are naturally occurring adenine derivatives whose physiological role is that of growth regulators in plants and that show also many other activities either in plants and in mammalian cells. In plants, they can be found mainly as free bases ((N^6-substituted adenines, CKs), but also as the corresponding N^9- ribosides (N^6-substituted adenosines, CKRs). In mammalian cells, CKRs are, in general, more active than CKs. In order to evaluate the intrinsic *in vitro* antioxidant capacity of some significant CKRs, their scavenging activity against synthetic radicals that are at the basis of well-established antioxidant assays (ORAC, TEAC, DPPH) has been evaluated. The results of the *in vitro* scavenging activity of biologically relevant radicals such as hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{\cdot -}$) and lipid peroxides ($R\text{-}OO^{\cdot}$) are reported and discussed.

Keywords: oxidative stress; cytokinins (bases and ribosides); *in vitro* antioxidant activity; radicals; chemical assays.

1. Introduction

Cytokinins are adenine derivatives substituted at the N^6-position (structure **1**, Figure S1) that naturally occur as free bases in plants where they physiologically exert a hormonal activity promoting cell division, growth and retardation of senescence, thus influencing almost each part of plant developmental stages (Mok and Mok, 2001). Due to their peculiar effect on cell division and differentiation in plant cells, cytokinins were also studied for their effect on the differentiation of human cells, both normal and malignant (Honma and Ishii, 2002). The N^9-ribosides of CKs (CKRs) showed a higher biological activity, so that became also potential candidates for treating a variety of cancers (Voller et al. 2017, 2019).

It has been proposed that the activities of cytokinins can be associated to their capacity to lower the oxidative stress interacting with biological regulators of oxidative stress (Othman et al. 2016; Hönig et al. 2018). An intrinsic antioxidant effect due to their structure cannot be excluded and this aspect has been recently investigated by Brizzolari et al. (2016) for four natural N^6-adenines (CKs, **1a-d**,

Figure S1). We have now evaluated the radical scavenging activity of the corresponding N^9-ribosides (CKRs, **2a-d**, Figure S1) by *in vitro* assays that quantify the attitude of the compounds to react with synthetic radicals (Figure S2) according to hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms (Huang et al. 2005), i.e. ORAC (HAT), TEAC (ET) and DPPH [mixed HAT/ET (Foti et al. 2004)] assays.

The *in vitro* the scavenging activity of CKRs **2a-d** (Figure S1) against biologically relevant radicals such as the hydroxyl radical ($HO^{\bullet}$) was established by the 2-deoxyribose (2-DR) degradation assay (Halliwell and Gutteridge, 1981; Aruoma, 1994). Finally, all CKs and CKRs were *in vitro* assayed against lipid peroxides (Lazarević et al. 2020) and superoxide anion radical ($O_2^{\bullet-}$, Yen and Duh, 1994).

2. Results and discussion

2.1. ORAC, TEAC and DPPH assays of CKRs

The results of ORAC assay for CKRs (Figure S3) show that, in the range of 1 μM – 5 μM, *p*-TR is the most active CKR, most likely for the presence of the phenolic OH group, which readily donates H-atom to AAPH peroxyl radical ($AAPH^{\bullet} = ROO^{\bullet}$) generated in the ORAC assay.

Other H-atom donors that could react with $ROO^{\bullet}$ are present in the structure of CKRs, but the N^6-H group should play a major role (Steenken, 1989). In CKRs, this NH group is bound to structurally diverse groups, thanks to which different reactivity with $ROO^{\bullet}$ can be observed (*p*-TR > KR > iPAR > BR).

In the TEAC assay, at a concentration range 0.5 μM to 5 μM, only *p*-TR exhibited a scavenging activity that was nearly identical to that of *p*-T (Brizzolari et al. 2016). The overall results obtained from the examined CKs and CKRs suggest that only the phenol moiety of *p*-T and *p*-TR can react with $ABTS^{\bullet+}$ radicals by an ET mechanism. Interestingly, in the concentration range 0.5 μM to 5 μM, the antioxidant capacity of *p*-T and *p*-TR is higher than that of Trolox (Figure S4). This could

be related to a limited steric accessibility of the bulky $ABTS^{\bullet+}$ radical to the congested 6-hydroxy group in Trolox, that is flanked by two methyl groups.

Finally, neither CKs nor CKRs showed any scavenging ability against $DPPH^{\bullet}$ in the maximum concentration ranges of reagents and substrate that could be reached in ethanol.

2.2. Scavenger activity of CKRs against chemically generated hydroxyl radicals HO and superoxide radical ($O_2^{\bullet-}$).

The reactivities of all four CKRs with the chemically generated hydroxyl radicals ($HO^{\bullet}$) were higher than that of adenosine (adenine riboside, AR, Figure S5), indicating an inductive effect of the N^6-substituents in the reactivity of the N^6-hydrogen (6-NH group) (Steenken, 1989). Differently from CKs (Brizzolari et al, 2016), all four CKS showed a similar activity that could be explained by the presence of the N^9-ribose moiety (a withdrawing group according to Steenken, 1989), able to counteract ("neutralize") the electron-donating effect of N^6-substituents.

Among CKs and CKRs, only iPAR was able to scavenge *in vitro* the $O_2^{\bullet-}$ radicals (Figure S7) although this activity was lower than that of caffeic acid, a well-known inhibitor of superoxide anions (Gülçin, 2006). These results are similar to those recently obtained by means of cellular assays by Dassano et al. (2014) suggesting that part of biological activity of CKs and CKRs may be associated to the structure-related antioxidant capacity of the compounds.

2.3. Scavenger activity of CKs and CKRs against lipid peroxyl radicals (LP-$OO^{\bullet}$).

Results for the *in vitro* scavenging activity of CKs and CKRs against lipid peroxyl radicals (LP-$OO^{\bullet}$) show that only *p*-T and *p*-TR were weak scavengers (concentration range 0.18 mM - 3.6 Mm). At concentrations below 1 mM, a somewhat stronger LP inhibition effect of *p*-TR in comparison with *p*-T can be observed (Figure S6).

This result suggests that the role of cytokinins in plant lipoperoxidation observed by some autors (Wang et al. 2003; Stoparić and Maksimović, 2008) is due more to some regulatory effect on

oxidative stress in cells than to an intrinsic capability of the molecules to react with the lipoperoxyl radicals.

3. Experimental

A detailed description, including Tables with calculated IC50 (not listed in the text) is provided in the supplementary material.

4. Conclusions

A few, representative cytokinin ribosides (CKRs, **2a-d**) were evaluated by ORAC and TEAC assays in order to examine their attitude to react with synthetic radicals according to HAT or ET mechanisms. The DPPH assay (mixed HAT/ET) showed no reaction of CKs and CKRs in our experimental conditions.

In vitro scavenging activity of chemically generated, biologically relevant radicals was examined and only a week inhibitory activity of *p*-T and *p*-TR against lipoperoxyl radicals (LP-OO$^{\bullet}$) could be detected whereas OH radical showed a similar reactivity with all CKRs and the superoxide radical $O_2^{\bullet -}$ reacted only with iPAR.

Our results should be integrated by cellular assays in order to show the effect of CKRs (and CKs) on the biological regulators of the oxidative stress.

Disclosure statement

No potential conflict of interests were reported by the authors.

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Evaluation of the radical scavenging activity of some representative isoprenoid and aromatic cytokinin ribosides (N^6-substituted adenosines) by *in vitro* chemical assays

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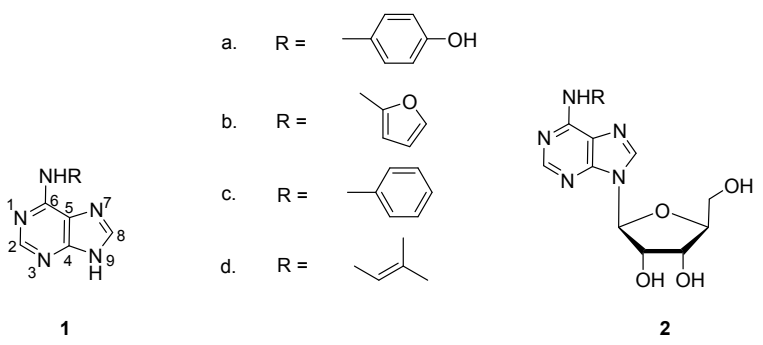
Supplementary material



CKs: N^6-4-hydroxybenzyladenine (*p*-topolin, **1a**, *p*-T), N^6-furfuryladenine (kinetin, **1b**, K), N^6-benzyladenine (**1c**, B), N^6-(Δ^2-isopentenyl)-adenine (**1d**, iPA)

CKRs: N^6-4-hydroxybenzyladenosine (*p*-topolin riboside, **2a**, *p*-TR), N^6-furfuryladenosine (kinetin riboside, **2b**, KR), N^6-benzyladenosine (**2c**, BR), N^6-(Δ^2-isopentenyl)-adenosine (**2d**, iPAR)

Figure S1. Chemical structure of cytokinin bases (CKs: iPA, B, K and *p*-T) and cytokinin ribosides (CKRs: iPAR, BR, KR and *p*-TR)

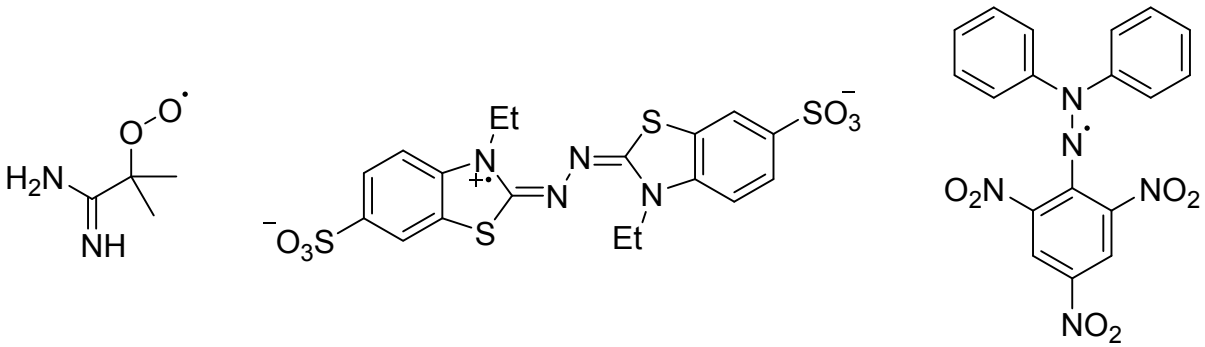


Figure S2. Chemical structures of radicals i) $AAPH^{\bullet}$, ii) $ABTS^{\bullet+}$ and iii) $DPPH^{\bullet}$ involved in ORAC, TEAC and DPPH assays.

1. Experimental section

1.1. Materials and Methods

CKs and CKRs were obtained from OlChemIm Ltd., (Olomouc, Czech Republic). All inorganic and organic reagents including the precursors of $AAPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, the stable DPPH radical and solvents were purchased from Sigma-Aldrich Co. (Milan, Italy). Phospholipids (Phospholipon® 90 G, unsaturated phosphatidylcholine content: 97.2%) was kindly provided as a gift by Phospholipid GmbH, Cologne, Germany.

The radicals involved in ORAC and TEAC assays ($APPH^{\bullet}$ and $ABTS^{\bullet+}$ were prepared according to the pertinent literature.

2-Amidinopropane-2-peroxyl radicals ($AAPH^{\bullet}$) were generated from the decomposition of commercially available 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) (Amorati and Valgimigli, 2015).

$ABTS^{\bullet+}$ radicals were generated by reacting ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] with potassium persulfate in buffered water just before the test (Re et al. 1999).

The experimental procedure for the ORAC, TEAC and 2-DR degradation were essentially conducted following the procedures developed in Brizzolari et al. (2016). The assay for the scavenging activity against DPPH was performed according to Lavelli et al (2009).

The lipid peroxidation scavenging assay was essentially conducted as described in Nazarević et al. (2020) and the superoxide $O_2^{\bullet-}$ was assayed according to Yen and Duh (1994).

All experiments were performed in triplicate.

As expected, the solubility of CKRs versus CKs was significantly different. Therefore, the process of solubilisation will be reported for all the assays.

1.2. IC_{50} calculation

The half maximal inhibitory concentration (IC_{50}) is a quantitative measure that indicates how much of a particular inhibitory substance (e.g. antioxidant) is needed to inhibit, *in vitro*, a given biological process or biological component (e.g. free radical) by 50%. However, from the curves

generated for the results of our experiments (except for the *p*-TR in TEAC assay) was not possible to calculate IC_{50} (EC_{50}) values since the concentrations in which we tested our compounds were lower than those required for the calculation of IC_{50}/EC_{50}. Regardless the mentioned experimental facts we went a step further in order to (wherever was possible) calculate the $IC_{50,}$ and to prepare CKR samples for new testing by adjusting the CKRs' concentration(s). The approximation we used to calculate the IC_{50} values per compound (CKR) affected by the radical involved in a particular assay was obtained by the calibration curves ($y = mx + q$) of assay, fixing $y = 50$ and resolving the curve equation. An explanation of why the calculated IC_{50} values remained the theoretical ones is what we have already stressed in the experimental part: the samples' solubility problems. To go further in testing higher concentrations was impossible because of the solubility limitations showed by CKRs in the media of the assays. Regardless of this fact, in the section of the corresponding experiment, the data related to the calculated theoretical IC_{50} values were also presented.

1.2. The ORAC assay

1.2.1. Reagent preparation

The solubility of CKRs in water:ethanol mixture (50:50) was complete after ultrasound treatment at room temperature (complete dissolution of CKRs required up to 10 min). The concentration range of CKRs was from 5 to 50 μM.

The data obtained by the ORAC assay were elaborated with GraphPad Prism version 6. The final results were presented by reporting Trolox equivalent versus concentrations of tested compounds (Figure S3), together with the theoretically estimated IC_{50} values, given in Table S1. As can be seen from the Table S1, IC_{50} values largely exceed initially tested concentration for all the compounds (CKRs).

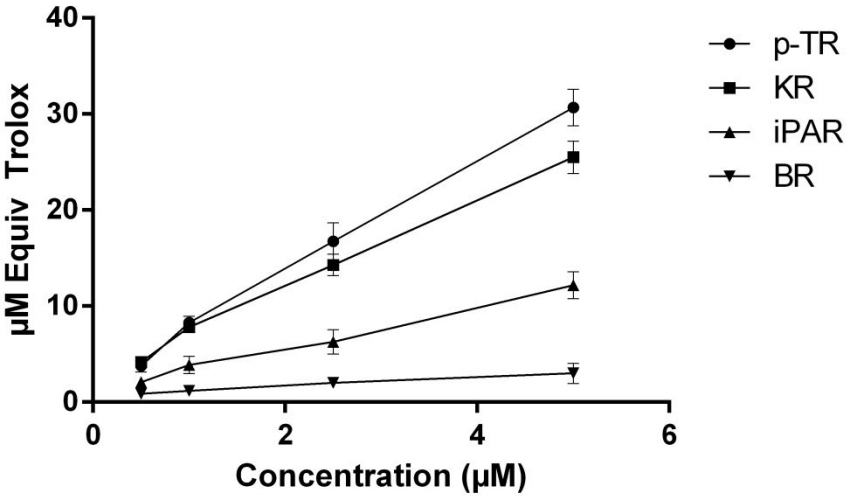


Figure S3. Peroxyl radical (AAPH• ORAC assay) scavenging activity of natural CKRs.

Table S1. ORAC IC_{50} of CKRs

Compound	**IC_{50} (µM)**
2a (*p*-TR)	8.26
2b (KR)	10.27
2c (BR)	n.d.[1]
2d (iPAR)	22.49

[1]not determinable because BR kinetic goes to plateau.

1.3. The TEAC assay

1.3.1. Reagent preparation

Solutions of CKRs 5 to 100 µM in ethanol were prepared just before the assay. Trolox was used as positive control in the same concentration range. The experimental results are reported in Figure S4 as percentage of inhibition of ABTS radical versus tested compound concentrations, whereas IC_{50} (for *p*-T theoretical) values are given in Table S2.

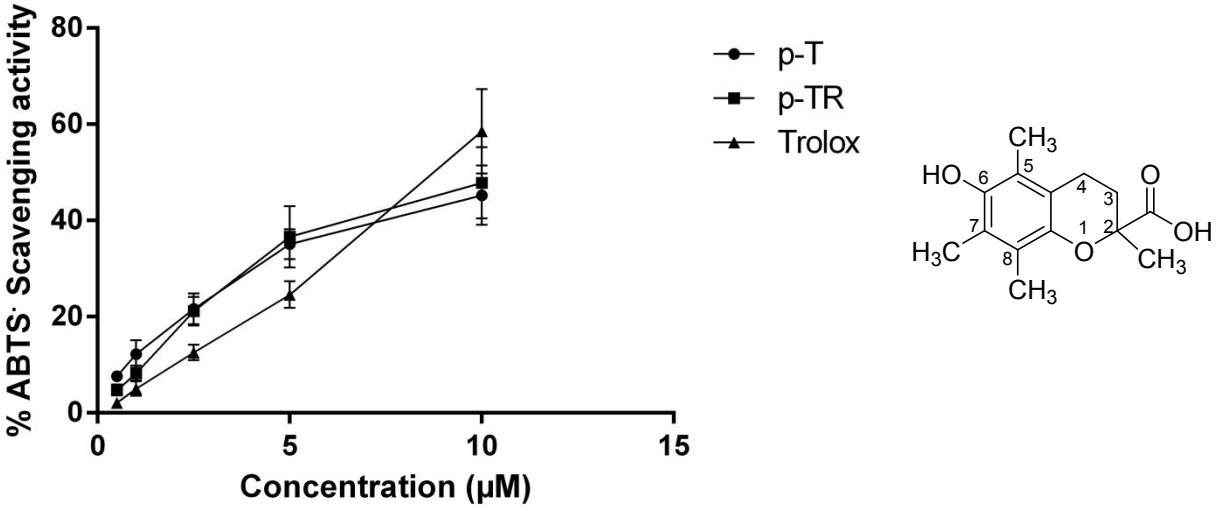


Figure S4. $ABTS^{\bullet+}$ scavenging activity of *p*-T and *p*-TR (TEAC assay) compared with Trolox.

Table S2. TEAC assay IC_{50}

Compound	**IC_{50} (μM)**
1a (*p*-T)	10.38
2a (*p*-TR)	9.56*
Trolox	8.80

* Not approximative, but exact IC_{50} value (determined from the experimental curve)

Note: other CKRs are not active

1.4. DPPH scavenging assay

1.4.1. Reagent preparation

Fresh solutions of $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl radical) were prepared by dissolving 7.5 mg of DPPH in 50 ml of ethanol (380.4 mM). The stock solution was stable for 24h hours (Otohinoyi et al. 2014) and the analytical sample was daily prepared by diluting an aliquot of the stock solution with absolute ethanol to 65 μM. Fresh solutions of CKs and CKRs (100 - 750 μM) were daily prepared dissolving the compounds in ethanol after ultrasound treatment (15 minutes)

at room temperature. The final concentration of the substrates in the assay was 8-65 µM ([DPPH•] = 65 µM, [CKs] and [CKRs] = 8 - 65 µM).

1.5. 2-Deoxyribose (2-DR) degradation assay

The protocol set up by Brizzolari et al (2016) for CKs was used for CKRs. These compounds were suspended in water and solubilised with ultrasound bath at the temperature of 50 °C for 10-15 minutes.

Results are expressed by reporting the percentage of scavenging activities versus tested compound concentrations (Figure S5).

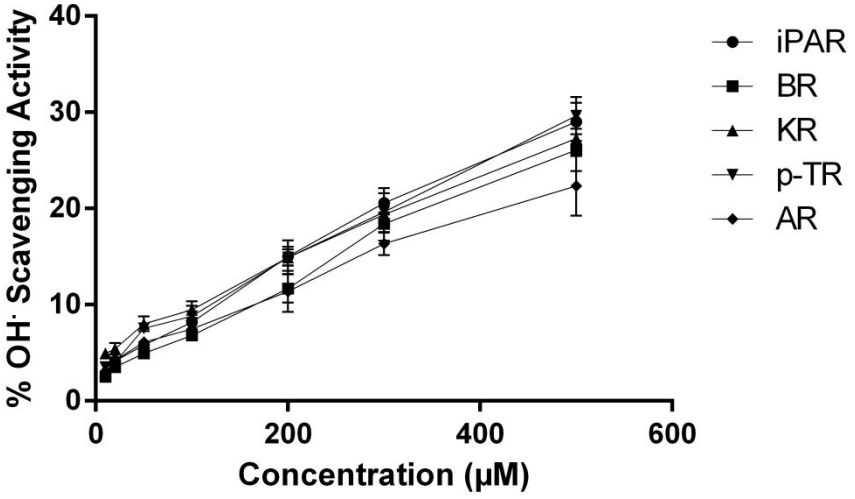


Figure S5. Hydroxyl radical scavenging activity of CKRs, compared to that of adenosine (AR).

Deoxyribose assay theoretically obtained IC_{50} values for CKRs are given in the Table S3. The IC_{50}, generated by approximative approach, are well beyond the tested concentrations.

Table S3. Deoxyribose assay IC_{50} of CKRs

Compound	**IC_{50} (µM)**
2a (*p*-TR)	878.29
2b (KR)	983.04
2c (BR)	978.28
2d (iPAR)	869.30

1.6. Lipid peroxidation inhibition

Lipid peroxidation (LP) and its inhibition in the presence of CKs and CKRs was evaluated according to the procedure described by Lazarević et al. (2020). Solutions of CKs/CKRs were prepared daily dissolving the compounds in methanol (concentration range 9 - 0.45 mM) after ultrasound treatment (10 minutes) at room temperature.

Results are expressed by reporting the percentage of LP inhibitory activities versus tested compound concentrations (Figure S6).

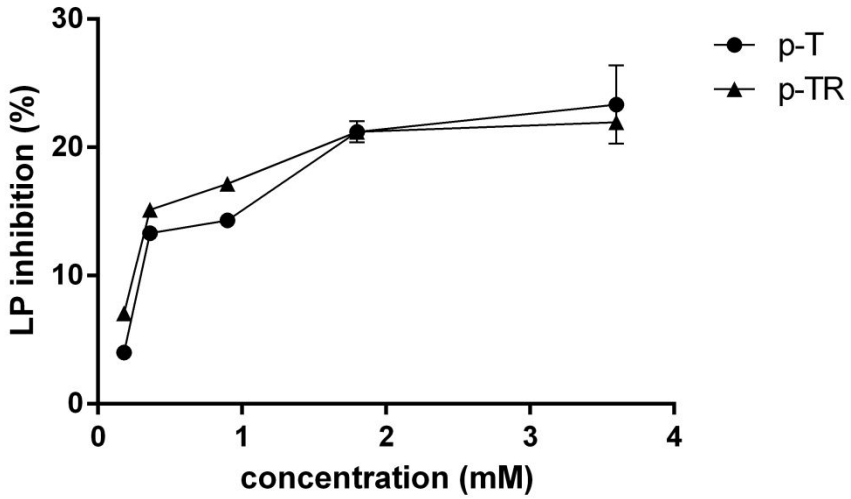


Figure S6. LP inhibitory activity of *p*-T and *p*-TR.

1.7. The superoxide anion assay

For the *in vitro* evaluation of the superoxide anions, we have selected the assay in which the chemical oxidation of NADH with phenazine methosulfate (PMS) generates superoxide radicals. Afterwards, the superoxide anions are oxidized by nitro blue tetrazolium (NBT) to form diformazan (Nishikimi et al. 1972).

In the presence of an antioxidant, the rate of NBT reduction decreases with respect to the blank and the method can be used for the evaluation of superoxide scavenging activity (Yen and Duh, 1994).

All CKRs were insoluble in the buffer solution used for the assay but were soluble in DMSO, that could be used for the assay since $O_2^{\bullet -}$ reacts with DMSO rather slowly and only at high DMSO concentrations (Herscu et al. 2008). The DMSO solution was added to the buffer in order to obtain the required, final concentrations (0.3 - 15 mM).

The measurements were performed in triplicate. Results are reported in Figure S7 as a percentage of inhibition of the superoxide anion versus tested compound concentrations compared to that of caffeic acid, a well-known inhibitor of superoxide anions (Gülçin, 2006).

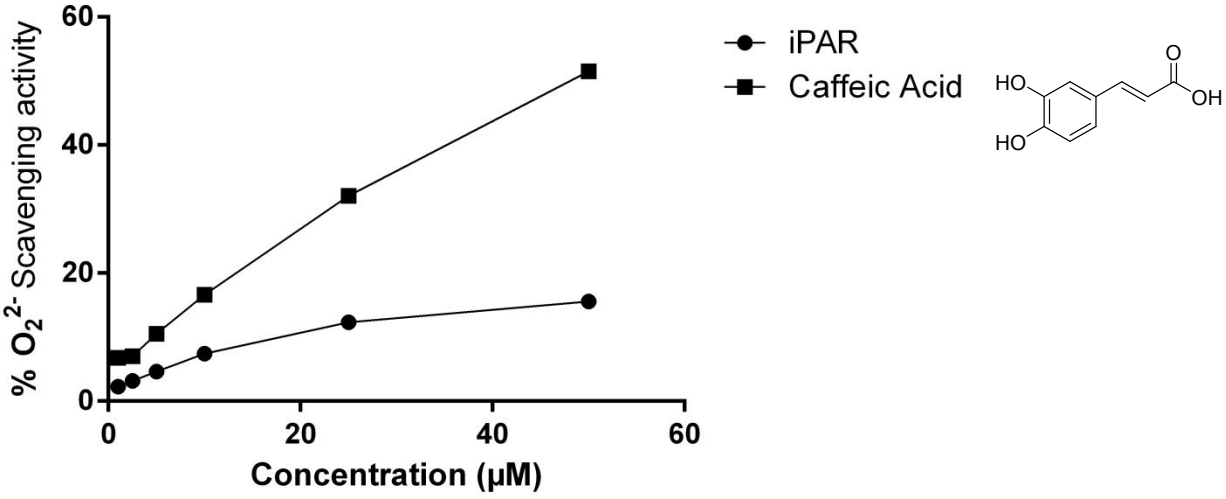


Figure S7. Superoxide anion scavenging activity of iPAR, and caffeic acid (positive control).

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