Antioxidant properties of hispidulin

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ABSTRACT
There are conflicting reports on the antioxidant activity of hispidulin. Antioxidant activity of hispidulin was evaluated using assays of ABTS\textsuperscript{*} reduction, ferric ion reducing antioxidant power (FRAP) assay, DPPH reduction assay, and protection of erythrocyte membranes against lipid peroxidation and protein thiol oxidation. ABTS\textsuperscript{*} reduction assay pointed to the involvement of all three phenol groups of hispidulin in ABTS\textsuperscript{*} reduction. The reactivity of hispidulin in the FRAP assay and DPPH reduction assay was low (0.09 and 0.019 of the reactivity of Trolox). However, hispidulin was effective in protection against erythrocyte membrane lipid peroxidation and highly effective in protection against erythrocyte membrane protein thiol group oxidation (more effective than Trolox). These results point to the necessity of caution in extrapolating the antioxidant activity evaluated in simple cell-free systems on more complex systems.

1. Introduction
Hispidulin (4', 5, 7-trihydroxy-6-methoxyflavone) (Supplementary material, Figure S1), a flavone present in plants of the Asteraceae and Lamiaceae families (Xu et al. 2009; Liu et al. 2020) is one of the most studied flavonoids. Research evidence shows that...
Hispidulin has a wide range of biological activities, including anti-inflammatory, anti-fungal, antiplatelet, anticonvulsant, antiosteoporotic, and notably anticancer activities (Patel and Patel 2017).

Hispidulin, like every flavonoid, is an antioxidant. However, the data on its antioxidant activity are relatively scarce and partly inconsistent. Hispidulin showed DPPH scavenging activity lower than but still comparable to nepetin and ascorbic acid and slightly lower antioxidant activity in a β-carotene-linoleic acid-assay than nepetin and rutin (Osman et al. 2014). Dabaghi-Barbosa et al. (2005) reported that hispidulin was incapable of donating electrons to reduce the stable free radical DPPH. However, Masood et al. (2018) reported good ability of hispidulin to scavenge DPPH, better than those of oxorilin A, baicalein and chrysin although the FRAP of hispidulin was lower in comparison with oxorilin A and baicalein. Part of these inconsistencies may be due to the fact that some studies were performed on hispidulin fraction of complex plant extracts, which might be not completely pure and include other compounds.

In view of controversial and incomplete data concerning the antioxidant properties of hispidulin and the potential usefulness of such data we examined the antioxidant activity of hispidulin in various experimental systems.

2. Results and discussion

We estimated the antioxidant activity of hispidulin in three single electron transfer reactions (SET): ABTS\(^\bullet\) reduction, FRAP and DPPH reduction. In all assays Trolox, also relatively hydrophobic antioxidant, was used as a comparative standard.

Both hispidulin and Trolox showed a linear dependence of the decrease of the decrease in ABTS\(^\bullet\) absorbance on the concentration, with slopes reported in Table S1 (Supplementary material). While the reaction of Trolox with ABTS\(^\bullet\) was completed within 1 minute, hispidulin exhibited both ‘fast’ and ‘slow’ reduction indicating the existence of a hydroxyl group/hydroxyl groups reacting with ABTS\(^\bullet\) at a lower rate.

In the assay of ABTS\(^\bullet\) reduction, the total reactivity of hispidulin with ABTS\(^\bullet\) was 2.84 higher than that of Trolox. Trolox has one phenol group; this result indicates that hispidulin has almost 3 phenol groups reactive with ABTS\(^\bullet\). For practical reasons, we terminated the reaction of hispidulin with ABTS\(^\bullet\) after 30 min, but the reaction of slow reduction could proceed further and reach the value closer or equal to 3, the number of phenol groups in the hispidulin molecule. The result of ‘fast reduction’ suggests that there is one fast reacting phenol group in the hispidulin molecule.

The reactivity of hispidulin in the FRAP assay was significantly lower in comparison with Trolox (Supplementary material, Figure S2). Comparison of the slopes of dependence of absorbance increase of TPTZ-Fe\(^{2+}\) complex on the concentration of hispidulin and Trolox indicates that the reductive reactivity of hispidulin for Fe\(^{3+}\) is 0.09 of that of Trolox. Comparison of the slopes of dependence of absorbance decrease on the concentration of hispidulin and Trolox shows that the reactivity of hispidulin for DPPH is 0.019 of the reactivity of Trolox (Supplementary material, Figure S3).

The much lower reactivity of hispidulin with respect to Trolox in the FRAP assay is surprising taking into account the similarity of the one-electron redox potentials of the ABTS\(^\bullet\)/ABTS redox couple (0.68 V) and the Fe(III)/Fe(II) redox couple (0.70 V).
difference may be due to the differences in pH in both assays (3.6 vs 7.4) and steric problems due to complexation of ferric ions with di-TPTZ in the FRAP assay (Müller et al. 2011).

The redox potential of the DPPH*/DPPH couple is much lower with respect to the ABTS*/ABTS and Fe(III)/Fe(II) couples. Cathodic and anodic peaks in electrochemical reduction of DPPH are equal to 251 mV and 310 mV, respectively (Milardović et al. 2006). Anodic peak potentials determined for flavones range from 0.40 (hesperidin and luteolin) to 0.70 V (diosmin) for Epa1 (Gil and Couto 2013) so they should be able to reduce DPPH. Perhaps there is a problem of steric hindrance again. The odd electron in the DPPH molecule is located mainly on the nitrogen hidden in the middle of the molecule, shielded by the phenyl and picrazyl moieties. Hispidulin molecule, apparently having steric problems with the reduction of Fe3+-di-TPTZ may have similar problems with the reduction of DPPH free radical.

Hispidulin was quite effective as inhibitor of peroxidation of erythrocyte membrane lipids, its efficacy being comparable to, though somewhat lower than that of Trolox (Supplementary material, Figure S4) and was effective in protection erythrocyte membrane protein thiol groups against oxidation, and was much more effective than Trolox (Supplementary material, Figure S5). This high efficiency of hispidulin in protection against thiol group oxidation may be due to its location in the membrane. Hispidulin molecules, like other flavonoids (Chaudhuri et al. 2007), can be expected to be located in the proximity of membrane proteins.

3. Conclusions

It is noteworthy that hispidulin, compound showing low antioxidant activity in the FRAP and DPPH assays is quite effective in a more complex, close to cellular system (erythrocyte membranes). It should be taken into account in extrapolation of antioxidant activity of various compound from results in simple cell-free systems; such extrapolations may sometimes be misleading.

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