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Effect of *O*-Glycosilation on the Antioxidant Activity and Free Radical Reactions of a Plant Flavonoid, Chrysoeriol

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Abstract—Chrysoeriol and its glycoside (chrysoeriol-6-*O*-acetyl-4′-β-D-glucoside) are two natural flavonoids extracted from the tropical plant *Coronopus didymus*. The aqueous solutions of both the flavonoids were tested for their ability to inhibit lipid peroxidation induced by γ-radiation, Fe (III) and Fe (II). In all these assays chrysoeriol showed better protecting effect than the glycoside. The compounds were also found to inhibit enzymatically produced superoxide anion by xanthine/xanthine oxidase system; here the glycoside is more effective than the aglycone. The rate constants for the reaction of the compounds with superoxide anion determined by using stopped-flow spectrometer were found to be nearly same. Chrysoeriol glycoside reacts with DPPH radicals at millimolar concentration, but the aglycone showed no reaction. Using nanosecond pulse radiolysis technique, reactions of these compounds with hydroxyl, azide, haloperoxyl radicals and hydrated electron were studied. The bimolecular rate constants for these reactions and the transient spectra of the one-electron oxidized species indicated that the site of oxidation for the two compounds is different. Reaction of hydrated electron with the two compounds was carried out at pH 7, where similar reactivity was observed with both the compounds. Based on all these studies it is concluded that chrysoeriol exhibits potent antioxidant activity. *O*-glycosylation of chrysoeriol decreases its ability to inhibit lipid peroxidation and reaction with peroxyl radicals. However the glycoside is a more efficient scavenger of DPPH radicals and a better inhibitor of xanthine/xanthine oxidase than the aglycone.

Introduction

Flavonoids are well known for their antioxidant activity, and are widely found in several plant and plant products. A good number of flavonoids have been successfully isolated and were found to show excellent antioxidant and many other pharmacological activities. So far at least 2000 varieties of flavonoids have been isolated from the plant products. Flavonoids that possess antioxidant activity are mostly polyphenolic compounds and a number of studies have been reported on the structure and activity of the flavonoids.[1–4] Chrysoeriol is one such flavonoid found in several tropical medicinal plants. In the literature, there are reports, where it has been isolated from different parts of the plants like Peanut hulls,[5] aerial part of *Cynanchum formosanum*,[6] *Stachys chrysantha* and *Stachys candida*.[7] It has also been isolated from the herbs of *Ludwigia prostrata* and flowers of *Lonicera japonica*,[8] leaves of *Epimedium sagittatum*[9] and from the plants of *Artemisia vulgaris*[10] etc. Its structure analysis has been reported by spectroscopic methods like NMR, IR, MS, melting point etc. However there are only a few reports in the literature on the antioxidant activity and almost no reports on the free radical scavenging ability. Chrysoeriol isolated from Peanut hulls has been tested for its ability to inhibit lipid peroxidation in low density lipoprotein induced by Cu^{2+}/O_2^- and the IC_{50} value was found to be 2.6 mM.[5] Chrysoeriol isolated from the aerial part of Gaiyou was found to exhibit antimutagenic activity in *Salmonella typhimurium* TA98.[11] Chrysoeriol isolated from *Morinda morindoide*s leaves has been found to be ineffective towards superoxide radicals generated from xanthine and xanthine oxidase.[12] Most dietary flavonoids occur as glycosides in nature.[13] The absorption, metabolism and antioxidant activity of flavonoids differ significantly with glycosylation and the properties vary with the position and nature of glycosylation.[4,14] Therefore, in this present study, antioxidant activity and free radical reactions of chrysoeriol and its glycoside (chrysoeriol-6-*O*-acetyl-4′-β-D- glucoside), isolated from the medicinal

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plant *Coronopus didymus* (family *Brassicaceae*), are reported. *Coronopus didymus* is an annual herb, a weed found in temperate and warm regions and belongs to the class of angiosperms. The antioxidant activity of these compounds has been examined by following the inhibition of γ-radiation, Fe(III) and Fe(II) induced lipid peroxidation in different model systems. The superoxide scavenging ability was studied by xanthine/xanthine oxidase method. Kinetics of superoxide reaction was determined by stopped flow spectrometer using superoxide as crown ether complex. In order to understand the ability to react with the free radicals and the mechanism underlying the free radical reactions, nanosecond pulse radiolysis technique was employed and the reaction of hydroxyl, peroxyl and other oxidising radicals with these compounds has also been studied in detail. The chemical structures of chrysoeriol and its glycoside are given in Scheme 1.

Chrysoeriol has 3 hydroxyl (OH) groups at 5, 7, and 4′ position, while the glycoside has 2 OH groups at 5 and 7 positions and the 4′-OH is *O*-glycosylated.

Results and Discussion

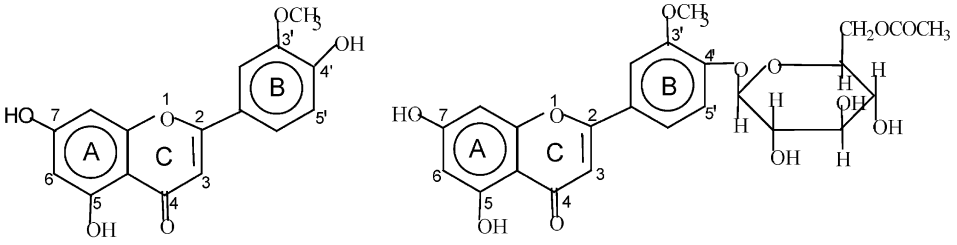
Lipid peroxidation

Figure 1a and b show the extent of lipid peroxidation in rat liver microsomes induced by γ-radiation in the absence and in the presence of 125 μM chrysoeriol respectively. The peroxidation increases with increasing dose from 136 Gy to 546 Gy (Gy is the unit for the absorbed dose in Gray; 1 Gy = 1J kg^{-1}). At 125 μM concentration, chrysoeriol showed significant inhibition of lipid peroxidation at all the doses. At a constant dose of 273 Gy, inhibition of peroxidation was followed at different concentrations of chrysoeriol. The TBARS formation was found to decrease with increasing chrysoeriol concentration from 50 μM to 198 μM (inset of Fig. 1) and the IC_{50} value, i.e., the concentraion at which the TBARS is inhibited by 50% was found to be 125 μM. Under similar conditions, 125 μM chrysoeriol glycoside showed only a negligible change in the peroxidation (not shown in the figure). Forty nine percent inhibition in TBARS formation was observed when the glycoside concentration was increased to 400 μM (absorbed dose = 273 Gy). At very high concentrations, the absorption of the glycoside interfered with the TBARS estimation and therefore could not be carried out.

We have also studied the effect of these compounds, on Fe(III) and Fe(II) induced lipid peroxidation in rat brain homogenates. Both microsomes and homogenates are commonly used for lipid peroxidation studies. Here brain homogenates are used as the liver microsomes generally contain nonheme iron, and may not act as suitable models for iron induced lipid peroxidation studies.[15] Figure 2a and b give the TBARS formation induced by Fe(III) in the presence of different concentrations of chrysoeriol and its glycoside respectively. Here also chrysoeriol showed better protection for Fe^{3+} induced lipid peroxidation than the glycoside derivative. The corresponding IC_{50} values are 90 μM and >400 μM, respectively, for chrysoeriol and the glycoside. Similarly Fe(II) induced peroxidation was also found to be inhibited much effectively by chrysoeriol as compared to the glycoside. At 400 μM concentration, chrysoeriol showed 80% reduction in TBARS formation, while the glycoside gives <30% inhibition. It has been reported that the flavonoid aglycones are more potent antioxidants than their glycosides.[16] Our results too indicate that chrysoeriol is a better inhibitor of lipid peroxidation than the glycoside. Some recent reports suggest that flavonoid glycosides undergo slow hydrolysis in small intestines and other cellular systems to give the corresponding aglycone.[17,18] Hence it is likely that such slow hydrolysis of chrysoeriol glycoside can take place in the microsomes and the homogenates, contributing to some extent in the antioxidant activity of the glycoside.

Reaction with superoxide radicals

We have studied the reaction of superoxide radical with chrysoeriol and its glycoside by two different methods. The enzymatically-produced superoxide radical from xanthine/xanthine oxidase was made to react with nitro blue tetrazolium (NBT) to give a characteristic absorption band (due to NBT^{+}) at 560 nm.[19,20] Reduction in the absorbance at 560 nm in the presence of different concentrations of chrysoeriol or the glycoside (Fig. 3a and b, respectively) was followed to estimate the reaction with superoxide radical. From this, the IC_{50} values were estimated to be 30 and 5 μM, respectively, for chrysoeriol and the glycoside. This clearly shows that



Chrysoeriol ***Chrysoeriol glycoside*** (chrysoeriol-6-*O*-acetyl-4'-β-D-glucoside)

Scheme 1.

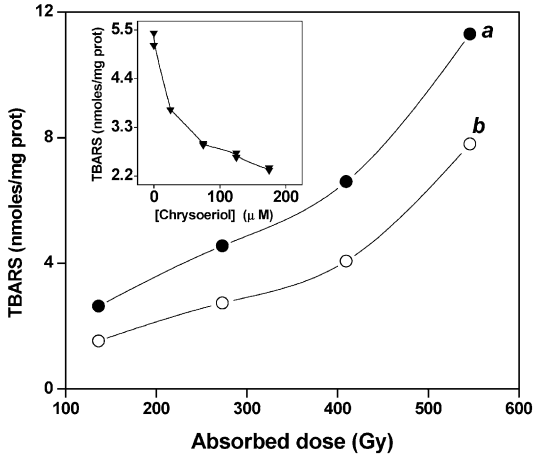


Figure 1. Effect of absorbed dose on radiation induced lipid peroxidation of rat liver microsomes at pH 7.4 (a) in absence and (b) in presence of 125 μM chrysoeriol. Inset shows effect of chrysoeriol concentration on radiation-induced lipid peroxidation (absorbed dose = 273 Gy).

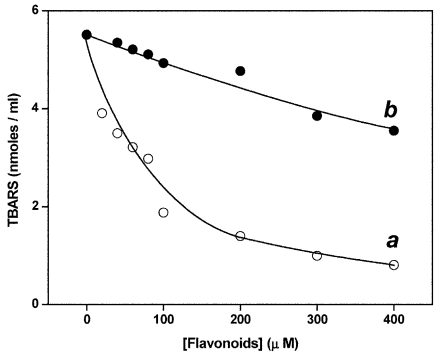


Figure 2. Effect of concentration of (a) chrysoeriol and (b) chrysoeriol glycoside on Fe^{3+} induced lipid peroxidation in rat brain homogenate [$FeCl_3$] = 100 μM, brain homogenate 10%.

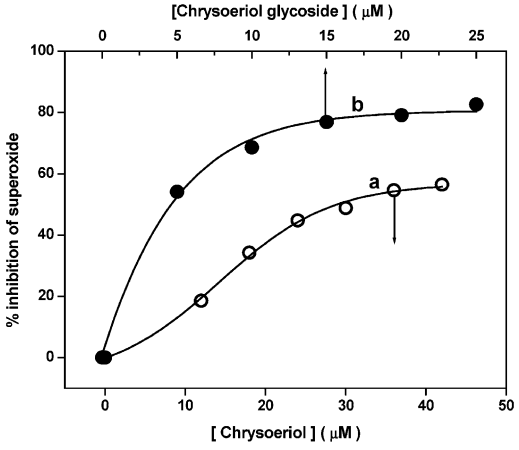


Figure 3. Percentage reduction in NBT^+ absorbance at 560nm as a function of (a) chrysoeriol and (b) chrysoeriol glycoside concentration. Experimental conditions: [Xanthine] = 50μM, [NBT] = 300μM, xanthine oxidase = 0.04 units/mL and [chrysoeriol] = 0–50μM, [glycoside] = 0–30μM in 20 mM phosphate buffer (pH 7.4).

the glycoside is more potent than the aglycone in inhibiting superoxide from these systems. Here, the activity can be either due to direct scavenging of the superoxide radical or by inhibiting the production of superoxide radical. In order to quantitatively evaluate the reactivity of these compounds with superoxide radicals, we have studied the kinetics of their reaction using stopped-flow spectrometer. For these studies, superoxide ion was prepared by solublizing KO_2 in dimethyl sulfoxide (DMSO) as crown ether complex.[21] The solubility and the stability of the superoxide were increased by such complexation with crown ether. In the stopped-flow cell, after mixing superoxide solution with either chrysoeriol or the glycoside, change in the absorbance at 360 nm (due to absorption by chrysoeriol and glycoside) was monitored as a function of time. Figure 4a and b show the absorption–time plots for the reaction of superoxide ion with chrysoeriol and its glycoside respectively. Fitting these signals to an exponential function, the first order rate constants (k_{obs}) were evaluated. This k_{obs} was found to depend linearly on the concentration of both flavonoid and superoxide radical. From the slope of the linear plot (Inset of Fig. 4a and b, respectively, for chrysoeriol and the glycoside), the bimolecular rate constant was evaluated to be $3.9 \pm 0.7 \times 10^3$ M^{-1} s^{-1} and $4.4 \pm 0.5 \times 10^3$ M^{-1} s^{-1}, respectively. From these studies, it can be concluded that the two compounds possess very similar reactivity with superoxide radical. Superoxide radical can cause both oxidation and reduction

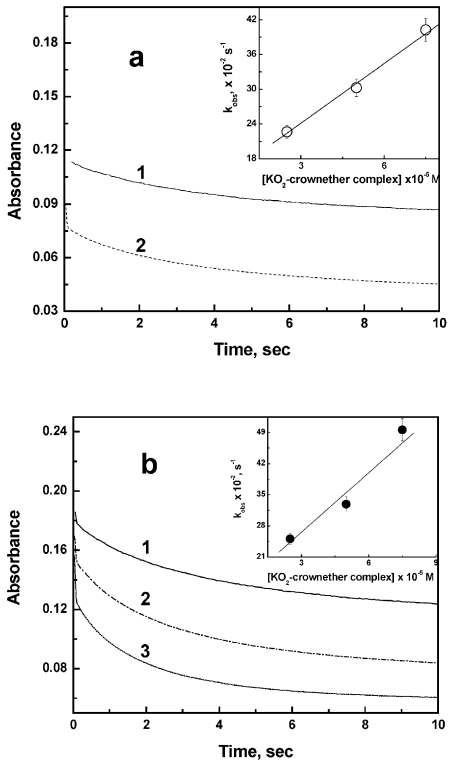


Figure 4. Absorption–time plots showing the decay of the absorption at 360 nm due to reaction of superoxide–crown ether complex with 15 μM of (a) chrysoeriol and (b) chrysoeriol glycoside. Plots 1 and 2 in (a) correspond to superoxide concentrations of 25 and 50 μM, respectively. Plots 1, 2 and 3 in (b) correspond to superoxide concentrations of 25, 50 & 75 μM respectively. Insets in a and b show variation of k_{obs} as a function of superoxide concentration.

and some times addition depending on overall energetics of the radicals involved.[2] The reaction of superoxide with flavonoids has been studied extensively and it has been proposed that the reactions take place by donating the unpaired electron from superoxide radical to the flavonoids.[2] Similar mechanism may be operating with these flavonoids also. From the above two experiments, it can be concluded that in addition to scavenging superoxide radicals, the glycoside may actually inhibit production of superoxide by inhibiting xanthine oxidase enzyme.

DPPH scavenging assay

The free radical scavenging activity of chrysoeriol and its glycoside was tested by employing DPPH radical scavenging assay.[4,22,23] DPPH is not a biologically important free radical. It is a stable free radical and its free radical character is neutralized in presence of molecules having ability to donate a hydrogen atom. It has often been used to estimate the antiradical activity of antioxidants. It has maximum absorption at 521 nm in DMSO ($\varepsilon_{max} = 10{,}980\ M^{-1}\ cm^{-1}$). In the presence of antioxidant substances, this radical is reduced to a colorless form. As a result of this, the absorption at 521 nm decreases. Earlier it has been reported[22] that chrysoeriol did not react with DPPH. Even in our present studies, after addition of millimolar concentration of chrysoeriol, no appreciable decrease in the absorption was found indicating that it is inactive in scavenging the DPPH radicals. However the glycoside though not very efficient, showed decrease in absorbance at 521 nm in the concentration range of 0.25–1 mM (Fig. 5). In flavonoids it has been observed that the DPPH activity is sensitive to the steric effects and also the planarity of the molecule.[4] In these present studies, our results on lipid peroxidation and DPPH activity of these compounds do not correlate. Such contradictory results have been found in literature and many flavonoid glycosides have been reported to be potent scavengers of DPPH than the aglycones.[4,23]

Pulse radiolysis studies

The reaction of important free radicals like hydroxyl and peroxyl radicals can be studied in short time scales (microseconds to millisecond) using pulse radiolysis technique. In this present study we have carried out reactions of these radicals with both chrysoeriol and its glycoside with an attempt to identify the nature and kinetics of free radical attack. Since reactions of these radicals with organic compounds are non-specific, studies have also been carried out with specific one-electron oxidants like $N_3^{\cdot}$ radicals. Unless otherwise mentioned, most of the pulse radiolysis studies have been carried out at pH 9.

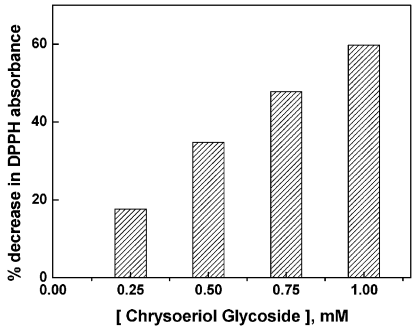


Figure 5. Effect of concentration of chrysoeriol glycoside on percentage decrease in DPPH (96 μM) absorption at 521 nm in DMSO.

The bimolecular rate constants for the reaction of $^{\cdot}OH$ radicals with these compounds were found to be within diffusion controlled limits and the rate constants are listed in Table 1. The transient spectra obtained on reaction of $^{\cdot}OH$ radicals with chrysoeriol and its glycoside is given in Figures 6a and 8a respectively. The spectra corrected for the ground state absorption are given in Figures 7a and 9a respectively. The corrected spectrum of the transient species formed on reaction of $^{\cdot}OH$ radical with chrysoeriol shows absorption bands at 330 and 390 nm with another broad absorption in 450–550 nm region (Fig. 7a). The reaction of the glycoside with hydroxyl radical shows peak near 390–400 nm with its tail extending beyond 500 nm and another strong and broad absorption near 310 nm (Fig. 9a). Hydroxyl radicals react with aromatic substrates by a number of reactions such as addition to the ring, one-electron oxidation and hydrogen abstraction.[24] In order to understand the nature of these transients and to determine the fraction of $^{\cdot}OH$ radical reacting by different channels,

Table 1. Bimolecular rate constants ($M^{-1}\ s^{-1}$) for the reaction of radiolytically generated radicals with chrysoeriol and its glycoside at pH 9 and 7

Radicals	Chrysoeriol (λ_{max}nm)[a]	Chrysoeriol Glycoside (λ_{max}nm)[a]
Hydroxyl (pH 9)	5.6×10^9 (330, 470)	1.2×10^{10} (330, 440)
Hydroxyl (pH 7)	1.3×10^9 (330, 470)	4.9×10^9 (300, 450)
$N_3^{\cdot}$ (pH 9)	3.7×10^9 (330, 470)	2.9×10^9 (330)
$CCl_3O_2^{\cdot}$ (pH 9)	2.1×10^8 (330)	2.0×10^7 (330)
e_{aq}^- (pH 7)	5.3×10^9 (375)	8.0×10^9 (375)

[a]Uncorrected spectra.

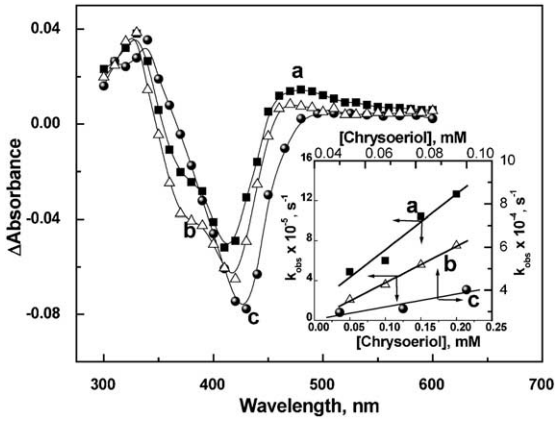


Figure 6. Difference absorption spectra of the transients for the reaction of pulse radiolytically generated (a) $^{\cdot}OH$, (b) $N_3^{\cdot}$ and (c) $CCl_3O_2^{\cdot}$ radicals with chrysoeriol at pH 9. Dose 15–17 Gy/pulse. Inset shows linear plots for the variation of observed pseudo first order rate constant (k_{obs}) for the reaction of (a) hydroxyl, (b) azide and (c) halogenated peroxyl radicals with chrysoeriol as a function of concentration.

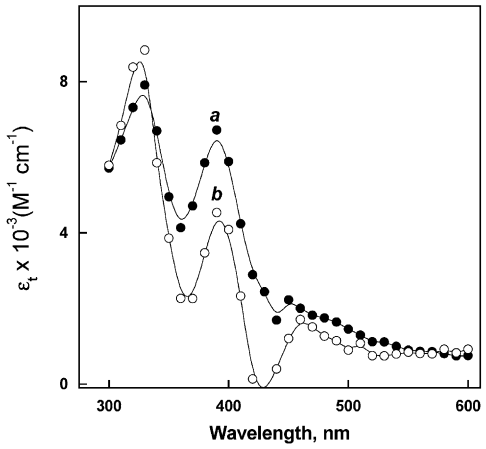


Figure 7. Corrected transient absorption spectra obtained on reaction of (a) $^{\cdot}OH$ and (b) $N_3^{\cdot}$ radicals with chrysoeriol.

reactions of these compounds were carried out with $N_3^{\cdot}$ radical, a specific one-electron oxidant.[25]

Pulse radiolysis of N_2O-saturated aqueous solution containing 0.05 M NaN_3 and 100 μM chrysoeriol and its glycoside at pH 9, generated transients with absorption spectra as shown in Figures 6b and 8b respectively. The corresponding spectra after correcting for parent absorption are shown in Figures 7b and 9b respectively. The bimolecular rate constants for the reaction of $N_3^{\cdot}$ radicals with both the compounds were determined (Table 1) by following change in the pseudo-first-order rate constant (k_{obs}) at 330 nm, as a function of parent concentration and are given in the inset of Figures 6 and 8 for chrysoeriol and glycoside respectively. As $N_3^{\cdot}$ radicals are specific one-electron oxidants their reaction will produce one-electron oxidation.[25] From the corrected spectra it can be seen that the one-electron oxidized chrysoeriol exhibits absorption bands at 330 and 390 nm (Fig. 7b), while that of the glycoside shows

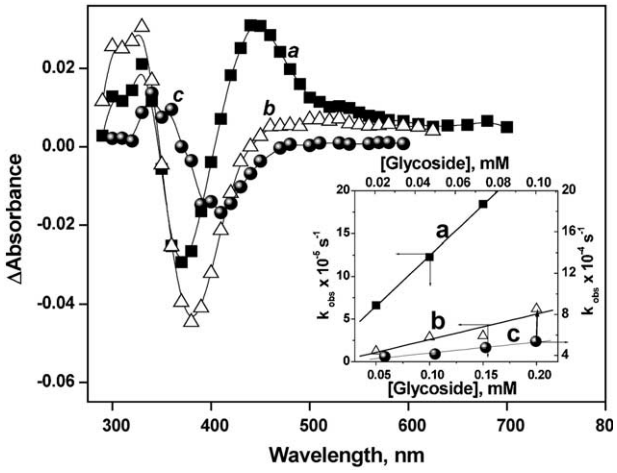


Figure 8. Difference absorption spectra of the transients for the reaction of pulse radiolytically generated (a) $^{\cdot}OH$, (b) $N_3^{\cdot}$ and (c) $CCl_3O_2^{\cdot}$ radicals with chrysoeriol glycoside at pH 9. Inset shows linear plots for the variation in observed pseudo first order rate constant (k_{obs}) for the reaction of (a) $^{\cdot}OH$, (b) $N_3^{\cdot}$ and (c) $CCl_3O_2^{\cdot}$ radicals with chrysoeriol glycoside as a function of the parent concentration.

absorption maxima at 310 and 390 nm (Fig. 9b). On comparing the spectrum of the oxidized species (Fig. 7b: chrysoeriol and Fig. 9b: glycoside) with that of the $^{\cdot}OH$ radical reaction (Fig. 7a: chrysoeriol and Fig. 9a: glycoside) it can be seen that the nature of the spectrum is similar, but the relative absorbance at 330 or 310 nm and 390 nm is different for the two reactions. The transient from $N_3^{\cdot}$ radical reaction shows higher absorption at 310–330 nm, while at 390 nm it is lower (Fig. 7b). Considering that the yield of the one-electron oxidized species formed on reaction with $N_3^{\cdot}$ radicals is 100%, and from the absorbance at 330 nm (chrysoeriol) and 310 nm (glycoside), the yield of one-electron oxidized species formed by hydroxyl radicals with chrysoeriol and glycoside at pH 9 are 89.5% and 94.5%, respectively. Remaining fraction of $^{\cdot}OH$ radicals may be reacting by adduct formation, which may be absorbing in the 370–500 nm region, thus increasing absorbance at 390 nm. The fact that the absorption maxima of the one-electron oxidized species of chrysoeriol (Fig. 7b) and its glycoside (Fig. 9b) are different suggests that the nature of oxidation on these two molecules is at different sites. In both the compounds ring A contains identical two phenolic OH groups, and their structures mainly differ in ring B. Chrysoeriol has a free hydroxyl group in the B ring, which is glycosylated in case of chrysoeriol glycoside. Reaction of $N_3^{\cdot}$ radicals with chrysoeriol will be on the hydroxyl OH group of the B ring, which produces phenoxyl radicals upon oxidation, in analogy with several other hydroxy flavones.[3] This phenolic OH is however unavailable for oxidation in glycoside. Therefore, oxidation is possible only with the phenolic OH on ring A, producing a different kind of phenoxyl radicals, that exhibit absorption maxima at 310 and 330 nm. The one-electron oxidized radicals of these compounds do not show any appreciable decay in the detectable timescales; hence no comparison of the decay kinetics of the transient species could be made. Based on these results it is concluded that the rate constants for the reactions of chrysoeriol and its glycoside with hydroxyl and $N_3^{\cdot}$ radicals are comparable and the structural changes mainly cause difference in the site of oxidation.

Peroxyl radicals are one of the biologically important free radicals produced during oxidative stress.[26] Their

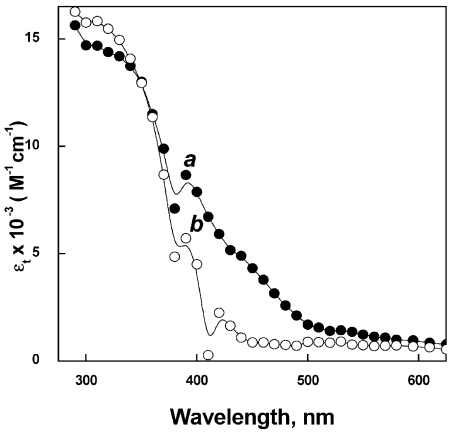


Figure 9. Corrected transient absorption spectra obtained on reaction of (a) $^{\cdot}OH$ and (b) $N_3^{\cdot}$ radicals with chrysoeriol glycoside.

production often leads to chain reactions and therefore these radicals are considered as more detrimental than many other free radicals. It is therefore essential for an antioxidant to scavenge these free radicals in order to act as a chain breaking antioxidant. Halogenated peroxyl radicals are model perxoyl radicals, which can be very conveniently produced by radiolysis.[27] We studied the reaction of $CCl_3O_2^{\cdot}$ radicals with these two compounds at pH 9. The transient absorption spectra are given in Figures 6c and 8c for chrysoeriol and its glycoside respectively. The parent absorption spectrum in isopropanol/CCl_4 matrix strongly interfered with the transient measurements in 300–500 nm region. Hence studies were restricted only to low concentration (50 μM) of the compounds, although at these concentrations, the reaction with peroxyl radicals is not complete. The bimolecular rate constants for the reactions of $CCl_3O_2^{\cdot}$ radicals with these compounds were also determined by following the pseudo-first-order rate constant at 330 nm at different concentrations of the parent (25–100 μM) and the values are listed in Table 1. At pH 9, chrysoeriol reacts with a rate constant of $2.1\pm0.8\times10^8$ M^{-1} s^{-1}, while at the same pH, the glycoside reacts with a rate constant $2.0\pm0.3\times10^7$ M^{-1} s^{-1}, which is less by an order of magnitude. This indicates that although the two compounds show similar reactivity with powerful oxidants, they differ in their reaction with less powerful oxidants like $CCl_3O_2^{\cdot}$ radicals. This may be one of the crucial factors deciding their antioxidant activity or the ability to inhibit lipid peroxidation. Reactivity towards peroxyl radicals is certainly more effective in inhibiting the chain reaction of peroxidation.[26] These results suggest that the OH group on the B ring is easily oxidizable as compared to the OH group on the A ring. Due to this, chrysoeriol is showing a higher rate constant value and so also the increased effect in the inhibition of lipid peroxidation.

The hydroxyl radical reaction product of chrysoeriol was also found to react with $ABTS^{2-}$ radicals producing the characteristic absorption band due to $ABTS^{-\cdot}$ at 645 nm.[28] For this reaction the concentration of chrysoeriol was kept as high as 1.6 mM and that of $ABTS^{2\cdot}$ was varied from 10 to 160 μM at pH 9. Under these conditions, $^{\cdot}OH$ radicals would react with chrysoeriol and not with $ABTS^{2-}$. From the observed pseudo-first-order rate constant the bimolecular rate constant for this reaction was determined to be 7.2×10^9 M^{-1} s^{-1}. These studies suggest that the one-elctron reduction potential of chrysoeriol is higher than that of $ABTS^{2\cdot}/ABTS^{-}$ couple (0.68V vs NHE).[28]

After understanding the oxidation behaviour, we tried to study the reduction of these molecules. One-electron reduction of these compounds was studied at pH 7 using hydrated electrons produced by the radiolysis of water. The transient absorption spectra obtained on reaction of $e^{\cdot}_{aq}$ with these two compounds are shown in Figures 10a and b respectively and the spectra after correcting for the ground state absorption in the inset of Figure 10. They show absorption maxima at 375 nm, however the extinction coefficients are significantly different. The bimolecular rate constants for the reaction

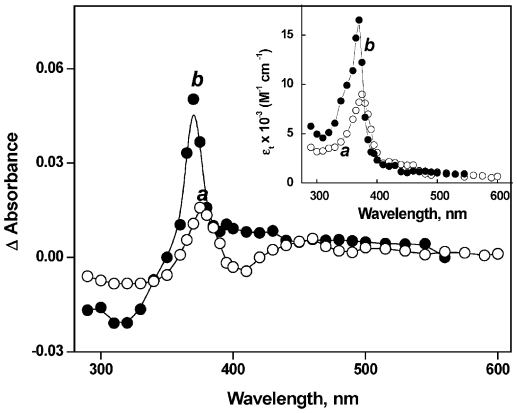


Figure 10. Difference transient absorption spectra obtained on reaction of e^{-}_{aq} with 100 μM (a) chrysoeriol and (b) chrysoeriol glycoside at pH 7. Inset shows the corrected transient spectra obtained on reaction of e^{-}_{aq} with 100 μM (a) chrysoeriol and (b) chrysoeriol glycoside.

of hydrated electron with these compounds have been determined by following the decay of $e^{\cdot}_{aq}$ in presence of different concentrations (50 μM to 100 μM, pH = 7) of the compounds and listed in Table 1. The preferential site of attack for the $e^{\cdot}_{aq}$ is the C=O group in the flavone ring to form a radical anion. Since the two molecules do not differ in the flavone structure, they produce similar transient absorption spectra on reaction with $e^{\cdot}_{aq}$. This particular study can be helpful in understanding the reaction of superoxide anion with the two compounds, where electron donation to the flavone ring is responsible for similar rate constants with the two compounds.

Conclusions

The ability of flavonoid antioxidant to inactivate oxidizing radicals is attributable to the presence of phenolic OH group.[1–4] There is a clear correlation between the position of OH group and their activity. Flavonoids having substitution of OH group in ring B and ring C as found in quercetin, catechin, etc exhibit very high antioxidant potential. Substitution of OH in 3′, and 4′ in the ring B makes them selective towards organic peroxyl radicals. Substitution of OH in 5 and 7 positions of ring A gives them a great capability to scavenge superoxide radicals.[29] Thus each flavonoid depending on the substitution shows unique activity towards various free radicals. Also most of the flavonoids are more abundant in nature as glycosides and the glycosilation of flavonoids changes its antioxidant activity. In the present investigation, we have studied using different assays, the antioxidant activity and the free radical scavenging ability of two compounds chrysoeriol and its *O*-glycoside and their properties were correlated with the molecular structure. Presence of OH group in 5 and 7 positions in both the compounds makes them excellent candidates for superoxide reaction.[29] However the *O*-glycosylation appears to have improved efficiency in inhibiting superoxide radicals generation probably by inhibiting xanthine oxidase activity. Similarly the reactivity of the compounds towards DPPH radicals is different. Here the glycoside reacts in millimolar concentration range while chrysoeriol is unreactive

towards DPPH radicals. Chrysoeriol having phenolic OH at 4′ position shows a greater activity towards peroxyl radicals like CCl_3O_2. But blocking the 4′ OH with glycosylation in chrysoeriol glycoside makes the reaction with peroxyl radical slower by more than ten times. As a result of this chrysoeriol shows better efficiency in inhibiting lipid peroxidation than the glycoside in microsomes and homogenates. Chrysoeriol also exhibits better protection than the glycoside towards Fe(III) induced lipid peroxidation, probably due to metal chelation. The one electron potential for the oxidation of chrysoeriol is >0.68 V, a value similar to that observed with other well-known flavonoids. The one electron potential for the oxidation of chrysoeriol glycoside appears to be more than that of chrysoeriol. Thus the two compounds differ significantly in the antioxidant activity and radical scavenging ability. The aglycone is a better scavenger of peroxyl radicals, undergoes easy electron transfer and exhibits greater ability to inhibit lipid peroxidation than the glycoside. On the other hand the glycoside shows activity towards DPPH radicals and is a potent inhibitor of superoxide production from Xanthine/xanthine oxidase system. Thus the natural flavonioid either in the free form or in the glycoside form shows activity under different conditions. Considering the fact that the bioavailability and absorption of the glycoside is more in nature and if it is hydrolyzed to the aglycone in the biological systems, this new flavonoid may have therapeutic potential in future.

Materials and Methods

Chemicals

*Iso*propanol, carbon tetrachloride (CCl_4), dimethyl sulfoxide (DMSO) and *tert*-butanol were of spectrograde purity and obtained from Spectrochem. India. 2,2′-Diphenyl picryl hydrazyl hydrate (DPPH), nitro blue tetrazolium (NBT), potassium superoxide, xanthine and xanthine oxidase were purchased from Aldrich Chemicals, USA. All other reagents were of highest purity. N_2 and N_2O gases used for degassing the samples were of Iolar grade and obtained from Indian Oxygen, India. The solutions were prepared in 'nanopure' water obtained from Millipore-A and freshly prepared solutions were used for each experiment. The pH of the solution was adjusted with Na_2HPO_4/KH_2PO_4 phosphate buffers. All solutions contained 8–10 mM phosphate buffer.

Extraction and isolation of chrysoeriol and its glycoside

The detailed procedure for the extraction, purification and characterization of chrysoeriol and its glycoside from the plant *Coronopus didymus* is given in ref 30.

The whole plant was shade dried and the ethanolic extract was prepared in a Soxhlet apparatus (1 kg of powder in batches of 500 g each in a Soxhlet apparatus with 95% ethanol for a period of 22 h, approximately 25 cycles). The extract was filtered hot under suction, concentrated in vacuo and the concentrated solution was evaporated at room temperature to a semisolid consistency (yield = 140g). The extract was fractionated using petrol ether, diethyl ether, ethyl acetate and *n*-butanol.

The diethyl ether fraction (4 g) when treated with a small quantity of methanol (2 mL), deposited a yellow solid, which was filtered and recrystalized from methanol as yellow fine micro crystals (Chrysoeriol). The filtrate was concentrated and subjected to column chromatography using silica gel (50 g) as stationary phase. Elution was started with chloroform followed by gradient mixtures of chloroform and methanol. The eluate [methanol: chloroform (3:2)] on concentration afforded Chrysoeriol (similar chromatographic behavior: PC-Whatmann No. 1; 15% acetic acid as mobile phase; visualization: exposure to ammonia).

The ethyl acetate fraction was treated with methanol (2 mL), which led to the deposition of the yellow solid, which was filtered and recrystalized from methanol as yellow microneedles (Chrysoeriol glycoside). The compounds were characterized by IR, NMR, melting point and elemental analysis.

Isolation of microsomes and rat brain homogenates

Rat liver microsomes were isolated from the liver of male albino wistar strain rats (180–200 g) as described earlier.[31,32] The protein was estimated by the Lowry method.[33] During the experiment microsomes were diluted with pH 7.4 phosphate buffer. Rat brain homogenates (10% w/v) were prepared in 150 mM KCl and centrifuged at $800\times g$ for 10 min.[34] For incorporation of the flavonoids into the microsomes or homogenates, flavonoid stock solutions were preapared initially by dissolving either in 2 mM Na_2HPO_4 (up to 250 μM) or in 0.1% cold NaOH (>250 μM) and immediately diluted to the required concentration with pH 7.4 phosphate buffer. The experiments were performed in the shortest possible time to avoid hydrolysis of the glycoside in the alkaline pH. The optical absorption spectra of the compounds did not show any significant change within the experimental time, indicating stability even in the alkaline pH.

Lipid peroxidation

The lipid peroxidation was initiated by the following methods.

(a) γ- Radiolysis. Lipid peroxidation (LPO) was carried out by the γ-radiolysis of rat liver microsomes using ^{60}Co γ-source with a dose rate of 9.1 Gy min^{-1} as measured by standard Fricke dosimetry. It was followed at different absorbed doses in N_2O/O_2-purged microsomal solution in absence and presence of the flavonoid at physiological pH 7.4 (phosphate buffer). The detailed methodology used in the lipid peroxidation is given in earlier references.[35,36] The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) using 15% w/v trichloroacetic acid, 0.375% w/v TBA, 0.25 N hydrochloric acid,

0.05% w/v BHT as TBA reagent measuring the absorbance at 532 nm ($\varepsilon_{532} = 1.56\times10^5\ M^{-1}\ cm^{-1}$).

(b) Fe(III) and Fe(II) induced lipid peroxidation.[37] Lipid peroxidation was also initiated by adding ferric chloride (100 μM)/ferrous sulphate (100 μM) to a mixture containing the rat brain homogenate (0.5 mL) and test compound in a total volume of 1.5 mL. The reaction mixture was incubated for 20 min, at 37 °C. After incubation the reaction was stopped by adding 2 mL ice-cold 0.25 M HCl and the malonaldehyde was estimated as (TBARS). Here brain homogenates were used as the liver microsomes generally contain nonheme iron, and may not act as suitable models for iron induced lipid peroxidation studies.

Superoxide scavenging assay

The reactivity of the flavonoids with superoxide radicals anions ($O_2^{\cdot-}$) was measured by the competition with NBT. Superoxide was generated enzymatically (xanthine/xanthine oxidase) according to the methods described earlier.[19,20] Mixture of 50 μM xanthine, 300 μM NBT, 0.04 unit/mL xanthine oxidase and 0–50 μM flavonoid in 20 mM phosphate buffer (pH 7.4) was incubated at 25 °C for 10 min and the $NBT^{\cdot+}$ formed was measured spectrophotometrically at 560 nm. Kinetics of reaction of the flavonoid with superoxide as crown ether complex in DMSO[21] was studied using single mixing stopped flow reaction analyzer Model SX 18 MV (Applied Photo Physics, U.K), with averaging facility and the reaction followed by optical absorption. Here syringe I contained chrysoeriol or glycoside in DMSO and syringe II contained DMSO solution of superoxide–crown ether complex. The two solutions were mixed with the help of pneumatic drive. The concentration of the reactants becomes half on mixing inside the stopped-flow cell. The rate of the reaction was determined by fitting the decay trace to a single exponential function.

DPPH scavenging assay

Ninety-six μM DPPH in DMSO was mixed well with the flavonoid solution in DMSO and kept in dark for 25 min. The absorbance at 521 nm was monitored in presence of different concentrations of flavonoid. Blank experiment is also carried out to determine the absorbance of DPPH before interacting with the flavonoid.

Pulse radiolysis studies

Pulse radiolysis experiments were carried out using high energy electron pulses (50 ns, 7 MeV) obtained from a linear electron accelerator. The transients were detected by kinetic spectrometry and the optical path length is 1 cm. Detailed description of the pulse radiolysis setup is given in references.[38,39] Aerated aqueous solution of 0.01 M KSCN was used for determining the dose delivered per pulse using $G\varepsilon_{500} = 21{,}520\ M^{-1}\ cm^{-1}$ for the transient $(SCN)_2^{\cdot-}$ species at 500 nm.[40] G denotes the number of species per 100 eV of absorbed energy (G = 1 corresponds to 0.1036 μmol J^{-1}) and ε is the molar absorptivity of $(SCN)_2^{\cdot-}$ species at 500 nm. The dose per pulse was close to 12–17 Gy. The bimolecular rate constants were determined from the linear regression plots of k_{obs} versus solute concentration for at least three independent experiments and the variation was within 10%.

Radiolysis of N_2-saturated neutral aqueous solution leads to the formation of three highly reactive species ($^{\cdot}H$, $^{\cdot}OH$, e_{aq}^-) in addition to the less reactive or inert molecular products (H_2, H_2O_2, H_3O^+).

$$H_2O \rightarrow {}^{\cdot}H, {}^{\cdot}OH, e_{aq}^-, H_2, O_2, H_3O^+ \quad (1)$$

The radiation chemical yield or G value are $^{\cdot}H$ (0.055), $^{\cdot}OH$ (0.28), e_{aq}^- (0.28). The reaction with $^{\cdot}OH$ radicals was carried out in N_2O-saturated solutions where e_{aq}^- is quantitatively converted to $^{\cdot}OH$ radicals with G ($^{\cdot}OH$) = 0.56.

$$N_2O + e_{aq}^- \rightarrow N_2 + {}^{\cdot}OH + OH^- \quad (2)$$

$N_3^{\cdot}$ radicals were generated by irradiation of N_2O-saturated 5×10^{-2} M NaN_3, where all the $^{\cdot}OH$ radicals were converted into $N_3^{\cdot}$ radicals with G = 0.6 μmol/J (eq 3) and were used for oxidation of the flavonoid at pH 7 and 9.

$$^{\cdot}OH + N_3^- \rightarrow N_3^{\cdot} + OH^- \quad (3)$$

The yield of phenoxyl radicals of the flavonoid generated in hydroxyl radical reaction was calculated at pH 7 and 9 assuming $N_3^{\cdot}$ reaction with flavonoids to be 100%. $CCl_3O_2^{\cdot}$ radicals were generated by the radiolysis of aerated aqueous solutions of 48% isopropanol, 4% carbon tetrachloride and 5 mM Na_2HPO_4 according to the equations below.[41]

$$(CH_3)CHOH + {}^{\cdot}H/{}^{\cdot}OH \rightarrow (CH_3)_2^{\cdot}COH + H_2/H_2O \quad (4)$$

$$(CH_3)_2CHOH + CCl_4 \rightarrow CCl_3^{\cdot} + (CH_3)_2CO + H^+ + Cl^{\cdot} \quad (5)$$

$$CCl_4 + e_{aq}^- \rightarrow CCl_3^{\cdot} + Cl^- \quad (6)$$

$$CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot} \quad (7)$$

The extinction coefficient of the flavonoid radical (ε_{fla}) at 330 nm produced in the $N_3^{\cdot}$ radical reaction was corrected for the parent absorption and calculated according to the standard procedure.[42] For all pulse radiolysis experiments, the concentration of the flavonoids was kept 110^{-4} M, except for the experiment with $CCl_3O_2^{\cdot}$ radical reaction the concenteration of both the flavonoids was 5×10^{-5} M.

The reaction with e^-_{aq} was carried out in N_2-saturated solution containing 10% *tert* butanol to scavenge $^{\cdot}OH$ radicals (eq 8).

$$(CH_3)_3COH + {}^{\cdot}OH \rightarrow {}^{\cdot}CH_2(CH_3)_2COH + H_2O \quad (8)$$

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