RESULTS
Cu,Zn superoxide dismutase (Cu,Zn SOD) obtained from Sigma, Aldrich, U.S.A. was used in all the experiments. In order to carry out the studies related to the interaction of flavonoids with Cu,Zn SOD it was essential to know certain physical properties of Cu, Zn SOD. The following sections describe some properties of Cu,Zn SOD.

**Effect of Temperature and Ionic Strength**

The purified Cu,Zn SOD exhibited a remarkable thermal stability at higher temperatures. When the enzyme was preincubated for one hour at different temperatures, the activity remained constant up to 60°C. The enzyme remained active up to this temperature and then its activity declined at higher temperatures (Figure 6). Similarly, in another experiment, the Cu,Zn SOD was incubated at 70°C for different time intervals, and the results are shown in Figure 7. As shown in the figure, the inactivation was started after 30 minutes and there was maximum inactivation after 2 hours incubation at this temperature. The effect of ionic strength on the Cu,Zn SOD is shown in Table 5. The enzyme exhibited the resistance to higher ionic strength as indicated by the unchanged enzyme units when the buffers of different molarities were used.

**Interaction of Cu,Zn SOD with 1, 10 Phenanthroline**

The aim of present investigations is to study the interaction of certain flavonoids with Cu,Zn SOD which is a primary important defensive enzyme to fight with certain diseases associated with oxidative stress. As the flavonoids interact with metal ions, it can be presumed that Cu(II) of Cu,Zn SOD may be involved in such type of interactions. In order to confirm the possibility, of metal chelation of Cu,Zn SOD, we have selected a metal chelating agent, 1,10 phenanthroline (OP) for our initial studies. The effect of 1,10 phenanthroline on the activity of Cu,Zn SOD is shown in Table 6. As evident from the table, 1mM concentration of 1,10 phenanthroline partially inactivated the enzyme. The inactivation of Cu,Zn SOD remained constant by increasing the molar concentration of enzyme at a fixed
Figure 6 Effect of different temperatures on Cu,Zn superoxide dismutase (Cu,Zn SOD) activity.

Appropriate volumes of enzyme prepared in 15% glycerol in 15mM potassium phosphate buffer pH 6.8 were incubated at the indicated temperatures for 60 min. After the incubation was over, the aliquots were chilled in ice and activity was determined as described in “Methods”
Figure 7 Thermal stability of Cu,Zn SOD

Enzyme solution diluted in 15mM potassium phosphate buffer containing 15% glycerol solution was incubated at 70°C. At the indicated time intervals appropriate volume of the aliquot was withdrawn, chilled quickly and the Cu,Zn SOD activity was determined by the standardized procedure.
Table 5  Effect of ionic strength on Cu,Zn SOD Activity

Appropriate volume of the enzyme was assayed in the sodium pyrophosphate buffer (pH 8.2) of the indicated molarities and enzyme units were calculated.

<table>
<thead>
<tr>
<th>Molarity (mM)</th>
<th>Enzyme units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>80</td>
<td>34</td>
</tr>
<tr>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>200</td>
<td>33</td>
</tr>
</tbody>
</table>
Table 6 Effect of 1,10 phenanthroline on the activity of Cu,Zn SOD

In a total reaction mixture of 40 μl containing indicated amounts of Cu,Zn SOD, 1mM 1,10 phenanthroline was added and the reaction mixture was incubated for 1hour at room temperature. After incubation period was over, suitable aliquots were withdrawn and enzyme activity was determined. The control set was also run without 1,10 phenanthroline and percent activity was determined. Each value represents the average of at least three experiments performed independently.

<table>
<thead>
<tr>
<th>Concentration of 1,10 phenanthroline (mM)</th>
<th>Molar concentration of Cu,Zn SOD (µM)</th>
<th>Percent Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>73</td>
</tr>
<tr>
<td>0</td>
<td>6.0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>6.0</td>
<td>73</td>
</tr>
<tr>
<td>0</td>
<td>9.0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>9.0</td>
<td>70</td>
</tr>
</tbody>
</table>
concentration of 1,10 phenanthroline. In order to confirm whether free copper affects the binding efficiency of OP to the Cu(II) of Cu,Zn SOD, the interaction of Cu,Zn SOD with OP was also investigated in presence of 1mM copper. The results are shown in Table 7. As evident from the table, there was no difference in the inactivation pattern of Cu,Zn SOD in presence and absence of free copper. The results of table 7, indicated that the Cu,Zn SOD activity was almost completely recovered in presence of uric acid in a reaction mixture containing Cu,Zn SOD, OP and copper chloride.

The SDS-Polyacrylamide gel electrophoretic pattern of the above reaction mixtures is shown in Figure 8. As shown in the figure, the recovery of the enzyme activity in presence of uric acid was confirmed by the intensity of the band in lane d. In order to study the interaction of 1,10 phenanthroline with some other protein, we have selected Bovine Serum Albumin (BSA) for our experiment. BSA was incubated with 1,10 phenanthroline in presence and absence of Cu(II). The results are shown in Figure 9, which suggested that there was degradation of protein in presence of both 1,10 phenanthroline and Cu(II), while 1,10 phenanthroline and Cu(II) alone were ineffective.

**Interaction of Cu,Zn SOD with flavonoids**

**Myricetin – Cu,Zn SOD**

Myricetin is a flavonol commonly found in wine, tea, fruits, berries and medicinal plants. It has anti-inflammatory activity and its therapeutic potential includes its role as an anticarcinogenic, antiviral and antimicrobial agent. It is not known whether myricetin modulates the activity of Cu,Zn SOD during its interaction with the enzyme, so we have carried out the studies demonstrating the modulation of Cu,Zn SOD activity in the presence of myricetin alone as well as in combination with certain metal ions. In the initial experiment, varying concentrations of myricetin were incubated with Cu,Zn SOD for 90 minutes and results are shown in Table 8. As evident from the table, there was a marginal
Table 7  Effect of CuCl₂ and uric acid on the 1,10 phenanthroline-Cu,Zn SOD interaction.

In a total reaction mixture of 60 µl containing 4 µM Cu,Zn superoxide dismutase, 1mM 1,10 phenanthroline, 1mM CuCl₂ and 0.5 mM uric acid were added in different sets of experiment and Cu,Zn SOD activity was determined. The percent remaining activity was determined by taking the control as 100%. Each value represents the average of at least three experiments performed independently.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Cu,Zn superoxide dismutase (4 µM)</th>
<th>1,10 phenanthroline (1mM)</th>
<th>CuCl₂ (1mM)</th>
<th>Uric acid (0.5 mM)</th>
<th>Percent Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>93</td>
</tr>
</tbody>
</table>
A total reaction mixtures of 60μl containing 4μM Cu,Zn SOD was incubated with 1 mM 1, 10 phenanthroline and 1mM CuCl₂ in presence of 0.5 mM uric acid for a long duration of 18 hours at 4 °C. After the incubation was over, samples were withdrawn from the reaction mixtures and were analyzed on SDS-Page. Staining was done by silver nitrate.

a) Cu,Zn SOD alone

b) Cu,Zn SOD treated with 1mM 1,10 phenanthroline

c) Cu,Zn SOD treated with 1mM 1,10 phenanthroline in presence of 1mM CuCl₂

d) Cu,Zn SOD treated with 1mM 1,10 phenanthroline and 1mM CuCl₂ in presence of 0.5mM uric acid
Figure 9 Effect of 1,10 phenanthroline on Bovine Serum Albumin (BSA) in presence of CuCl₂.

In a reaction volume of 50µl, 58µM BSA (at final concentration) was treated with 1mM 1,10 phenanthroline in presence and absence of 1mM CuCl₂, and incubated for 2 hours at room temperature. Proper aliquots (≈10 µg protein) were withdrawn from the reaction mixtures and loaded on 7.5% native polyacrylamide gel. Finally, the staining was done by silver nitrate.

a  BSA treated with 1,10 phenanthroline

b  BSA control

c  BSA treated with CuCl₂

d  BSA treated with 1,10 phenanthroline in presence of CuCl₂
Table 8 Effect of Myricetin on Cu,Zn SOD activity.

A total reaction mixture of 67μl containing 7.5μM, Cu,Zn SOD and varying concentration of myricetin was incubated for 90 minutes at 37°C. After the incubation period was over, suitable aliquots were withdrawn for measuring Cu,Zn SOD activity as described in the text. Each value represents the average of at least three experiments performed independently.

<table>
<thead>
<tr>
<th>Concentration of myricetin (mM)</th>
<th>Percent Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>45.3</td>
</tr>
<tr>
<td>0.5</td>
<td>51.0</td>
</tr>
<tr>
<td>1.0</td>
<td>56.0</td>
</tr>
<tr>
<td>2.0</td>
<td>53.0</td>
</tr>
</tbody>
</table>
increase in the inactivation of Cu,Zn SOD by increasing myricetin concentrations from 0.1 -0.5mM and beyond this concentration, the enzyme inactivation pattern remained constant up to a maximum of 2mM concentration of myricetin used in this experiment. By lowering the concentration of myricetin and increasing the time of incubation, a different inactivation pattern of Cu,Zn SOD was obtained. The effect of varying concentration of myricetin 20-1000μM (at lower range) on Cu,Zn SOD activity are shown in Table 9. As shown in the table, 20μM concentration of myricetin decreased the Cu,Zn SOD activity upto 36% during 3 hours incubation and maximum inactivation, close to 80% was achieved at maximum concentration of myricetin ie. 1mM taken for this experiment. When 1mM myricetin was incubated with varying concentrations of Cu,Zn SOD (2.2-7.5μM), at 37°C for 90 minutes, maximum inactivation was observed at 4.4μM concentration of Cu,Zn SOD which remained unchanged at 7.5μM concentration of enzyme (Table 10). For our future studies we have considered this concentration of Cu,Zn SOD. The data obtained from these experiments, indicated that inactivation of Cu,Zn SOD was increased by increasing the time of incubation with myricetin. In order to optimize the time of incubation for maximum possible inactivation of Cu,Zn SOD, we have carried out an experiment by incubating a fixed concentration of myricetin with Cu,Zn SOD for different durations. The results are shown in Figure 10. As evident from the figure, at 1mM concentration of myricetin, there was a gradual decrease in the Cu,Zn SOD activity by increasing the time of incubation up to 3 hours as expressed in terms of increase in the percent inactivation. After 3 hours incubation, Cu,Zn SOD lost its almost 80% activity which remained constant up to 6 hours of incubation with the same concentration of myricetin. SDS-Polyacrylamide gel electrophoresis of myricetin treated Cu,Zn SOD and its respective control was carried out in order to monitor the fragmentation of the enzyme in association with the loss in enzyme activity. As shown in Figure 11, the myricetin treated Cu,Zn SOD activity exhibited an almost identical SDS gel electrophoretic pattern with respect to its control.
Table 9  Effect of myricetin on Cu,Zn SOD activity.

In a total reaction mixture of 67μl containing 7.5μM Cu,Zn SOD in 1.5mM potassium phosphate buffer pH 6.8 was incubated with varying concentrations of myricetin for 3 hrs at 37°C. After the incubation period was over, suitable aliquots were withdrawn for measuring Cu,Zn SOD activity by the method described in the text. Each value represents the average of at least three experiments performed independently.

<table>
<thead>
<tr>
<th>Concentration of myricetin (μM)</th>
<th>Percent Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>20</td>
<td>34.6</td>
</tr>
<tr>
<td>50</td>
<td>56.2</td>
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<tr>
<td>100</td>
<td>54.2</td>
</tr>
<tr>
<td>1000</td>
<td>78.2</td>
</tr>
</tbody>
</table>
Table 10 Effect of myricetin on Cu,Zn SOD activity as a function of enzyme concentration.

In a total reaction mixture of 67μl, containing 1mM myricetin in 1.5mM potassium phosphate buffer pH6.8, increasing concentrations of Cu,Zn SOD was added and reaction mixture was incubated at 37°C for 90 min. After the reaction period was over, Cu,Zn SOD was determined by the procedure described in the text. Each value represents the average of at least three experiments performed independently.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Percent Inactivation</th>
<th>Concentration of Cu,Zn SOD (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu,Zn SOD control</td>
<td>0.00</td>
<td>2.2</td>
</tr>
<tr>
<td>Cu,Zn SOD treated with 1mM myricetin</td>
<td>48.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Cu,Zn SOD control</td>
<td>0.00</td>
<td>4.4</td>
</tr>
<tr>
<td>Cu,Zn SOD treated with 1mM myricetin</td>
<td>67.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Cu,Zn SOD control</td>
<td>0.00</td>
<td>7.5</td>
</tr>
<tr>
<td>Cu,Zn SOD treated with 1mM myricetin</td>
<td>67.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>
Figure 10 Effect of time of incubation on Cu,Zn SOD activity in presence of myricetin.

The total reaction mixture of 67μl containing 1mM myricetin, 7.5μM Cu,Zn SOD in 15mM potassium phosphate buffer, pH 6.8 was incubated at 37°C for different time intervals and Cu,Zn SOD activity was determined as described in the text.
Percent remaining activity vs. Time of incubation (minutes)
Figure 11 SDS-Electrophoretic pattern of Cu,Zn SOD treated with myricetin.

Cu,Zn SOD at a final concentration of 7.5μM was incubated with 1mM myricetin in 1.5mM potassium phosphate buffer pH 6.8 at 37°C for 3 hrs. A control set containing enzyme alone was also incubated under similar conditions. The volume of the control set was compensated with the same buffer, and after the incubation period was over, suitable aliquots were withdrawn and analyzed by SDS-PAGE. Staining was done by silver nitrate method as described in the text.

a - Marker Protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with 1mM myricetin
From the above mentioned experiments, it was not known whether the myricetin induced inactivation of Cu,Zn SOD was reversible or irreversible in nature and required the presence of myricetin. In order to confirm this, the myricetin treated Cu,Zn SOD and its control were dialyzed extensively against 1.5mM potassium phosphate buffer (pH 6.8) before measuring the enzyme activity. The results are shown in Table 11. The results of Table 11 clearly indicated that dialyzed and undialyzed untreated/myricetin treated Cu,Zn SOD exhibited almost similar activity, maintaining nearly similar inactivation pattern. When these samples were also electrophoresed on SDS gel, there was no alteration in the electrophoretic pattern of dialyzed and undialyzed, myricetin treated as well untreated samples of Cu,Zn SOD (Figure 12).

Spectral studies on Myricetin – Cu,Zn SOD complex

In order to monitor the structural and conformational changes in the protein, during interaction with other molecules, various spectral studies are conducted. We have carried out the absorption, fluorescence, circular dichroism (CD) spectroscopy of myricetin treated Cu,Zn SOD. Figure 13 shows the UV/visible absorption spectra of untreated dialyzed Cu,Zn SOD control as well as myricetin treated Cu,Zn SOD samples. A slight shift towards the ultra violet region (hypsochromic shift), and a considerable decrease in the absorbance of myricetin treated Cu,Zn SOD was observed. The fluorescence emission spectra of control and myricetin treated Cu,Zn SOD exhibited an emission maxima at 295 and 292nm respectively (Figure 14) when excited at 280nm. Treatment of Cu,Zn SOD with 1mM myricetin also resulted in 40% decrease in the fluorescence intensity. Circular dichroism predominantly monitors secondary structures of proteins and polypeptides in the far UV spectral region (190-250nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Pure α helices show negative ellipticity at around 222 nm and at around 205 nm whereas pure β sheets generally show a shallow minima CD (Greenfield and Fasman, 1969). Figure 15, shows the CD spectra in the far UV region of dialyzed untreated / myricetin treated Cu,Zn SOD.
Table 11 Effect of dialysis on Cu,Zn SOD activity after treating with myricetin.

7.5μM solution of Cu, Zn SOD was incubated with 1mM myricetin at 37°C for 3 hours. A control set of enzyme (Cu,Zn SOD) alone in 1.5mM potassium phosphate buffer, pH 6.8 was also incubated under similar conditions. After the incubation period was over, half of the samples (both from the control and treated sets) were dialyzed extensively against 1.5mM potassium phosphate buffer, pH 6.8. Suitable aliquots from another half was taken for determining the Cu,Zn SOD activity and designated as undialyzed sample. After extensive dialysis, suitable aliquots were withdrawn for determining Cu,Zn SOD activity and samples were designated as dialyzed sample.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Percent Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dialyzed</td>
</tr>
<tr>
<td>Control Cu,Zn SOD</td>
<td>100</td>
</tr>
<tr>
<td>Cu,Zn SOD treated with 1mM myricetin</td>
<td>27.8</td>
</tr>
</tbody>
</table>
Figure 12 SDS gel electrophoretic pattern of Cu,Zn SOD treated with myricetin before dialysis and after dialysis.

The reaction mixture containing 7.5μM Cu,Zn SOD was treated with 1mM myricetin and incubated for 3 hrs at 37°C. After the incubation period, half of the reaction mixture was dialyzed extensively against 1.5mM potassium phosphate buffer, pH 6.8. Proper volume of aliquots (≥20μg protein) from both dialyzed and undialyzed samples were used for SDS-PAGE. Staining was done by silver nitrate method as described in the text.

a - Myricetin treated Cu,Zn SOD (dialyzed)

b - Cu,Zn SOD control (dialyzed)

c - Myricetin treated Cu,Zn SOD (undialyzed)

d - Cu,Zn SOD control (undialyzed)
Figure 13 UV/VIS spectral analysis of interaction of Cu,Zn SOD with 1mM Myricetin.

The reaction mixture containing 1mM myricetin and 7.5µM Cu,Zn SOD was incubated for 3 hrs at 37°C. The control sets containing Cu, Zn SOD and myricetin alone at similar concentration were also incubated under similar conditions. The volume in the control sets was compensated with 1.5mM potassium phosphate buffer, pH 6.8. After the incubation period, the reaction mixtures were extensively dialyzed against 1.5mM potassium phosphate buffer pH 6.8. Proper aliquots (± 56µg/ml) were withdrawn from the dialyzed samples and diluted in the same buffer. The diluted samples were used for recording the spectra.

Cu,Zn SOD control (.........)

Cu,Zn SOD treated with myricetin (———)
Figure 14 Changes in the fluorescence spectra of Cu, Zn SOD after interaction with 1mM myricetin.

Reaction mixture containing 7.5μM Cu, Zn SOD and 1mM myricetin was incubated at 37°C for 3 hours. The control sets containing Cu,Zn SOD and myricetin alone at same concentrations were also incubated under similar conditions. The volume in the control sets was compensated by adding 1.5mM potassium phosphate buffer, pH 6.8. After the incubation period, the reaction mixtures were dialyzed extensively against 1.5mM potassium phosphate buffer pH 6.8. Proper aliquots were withdrawn from the dialyzed samples (~ 56μg protein/ml) and diluted in the same phosphate buffer for the fluorescence spectra. The excitation wavelength was 280nm and scanning wavelength range was 250 nm-400nm.

1. Cu,Zn SOD control

2. Cu,Zn SOD treated with myricetin
In a total reaction volume of 1ml containing 7.5μM Cu,Zn SOD was incubated with 1mM myricetin for 3 hours at 37°C. Untreated Cu,Zn SOD at the same concentration was also incubated under similar conditions. After the incubation was over, the samples were dialyzed extensively against 1.5mM potassium phosphate buffer pH 6.8. Suitable aliquots from the dialyzed samples were withdrawn (≈100μg protein) and used for taking the spectra using the cells with 0.1 cm path length. The unit of the ordinate is given in CD (m deg).

Control Cu,Zn SOD (······)

Myricetin treated Cu,Zn SOD (- - -)
The spectra for Cu,Zn SOD is characterized by the presence of minima at 210nm and interaction with 1mM myricetin resulted in 2nm blue shift, while there was a marked decrease in the C. D. (mdeg) value with a change in the shape of the spectra.

A significant decrease in the Cu,Zn SOD activity in presence of 1mM myricetin clearly indicated the enzyme inactivation by an unknown mechanism. It has already been observed that the inactivated Cu,Zn SOD recovered in the presence of excess amount of Cu(II) (Dameron and Harris, 1987). We have carried out one experiment in which myricetin treated and dialyzed Cu,Zn SOD was incubated with varying concentrations of CuCl₂ (10-100μM) (Table 12). As evident from the table, no recovery in the Cu,Zn SOD activity was achieved at any of the concentrations used in our experiment.

**Studies on reduction of Cu(II) → Cu(I) of Cu,Zn SOD in presence of myricetin**

In order to explore the mechanism of Cu,Zn SOD interaction with myricetin and the inactivation of enzyme involving its Cu(II), we have carried out stoichiometric titration of Cu(I) production. When 22.5μM concentration of myricetin was incubated with varying concentrations of Cu(II), its reduction into Cu(I) exhibited a linear range of absorbance in presence of neocuproine. The results are shown in Figure 16. When Cu,Zn SOD was added to the reaction mixture containing myricetin and neocuproine, the change in absorbance indicated the reduction of Cu(II) of Cu,Zn SOD into Cu(I). The low values of Cu(II):myricetin ratio in case of Cu,Zn SOD determined from the standard plot, was 3.3x10⁻⁴. These low values, were obtained due to low concentration of enzyme taken for this experiment.

**Interaction of Cu, Zn SOD with myricetin involving Cu(II)**

Since it is known based on certain observations that flavonoids may also act as a prooxidant in presence of certain metal ions (Cao, et al., 1997; Cotelle, 2001). In order to see the interaction of Cu,Zn SOD with myricetin in presence of Cu (II) as
Table 12 Effect of Cu$^{2+}$ on myricetin treated and dialyzed Cu,ZnSOD.

$7.5 \mu$M Cu,Zn SOD alone and with 1mM myricetin samples in a total reaction mixture of 1ml were incubated at 37°C for 3 hours. After incubation period was over, both the samples were dialyzed extensively against 1.5mM potassium phosphate buffer pH 6.8 and suitable volume of aliquots were withdrawn from control (referred as A) as well as from myricetin treated (referred as B) sets. These aliquots were incubated with varying concentration of CuCl$_2$ at 37°C for 3 hrs. The Cu,Zn SOD activity was determined in each sample. The value of untreated and dialyzed Cu,Zn SOD control was considered as 100% for calculating percent remaining activity. Each value represents mean of three different experiments performed in duplicates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration of CuCl$_2$ (μM)</th>
<th>Percent Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>0.0</td>
<td>28.7</td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>105</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>23.9</td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>104</td>
</tr>
<tr>
<td>B</td>
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</tr>
<tr>
<td>A</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>27</td>
</tr>
</tbody>
</table>
Figure 16 Stoichiometric titration of reduction of Cu(II) $\rightarrow$ Cu(I) of Cu,Zn SOD in presence of myricetin.

The total reaction of 3ml containing 0.4mM neocuproine, 22.5$\mu$M myricetin was prepared in 10mM Tris HCl buffer (pH 7.5) and absorbance was recorded at 450nm after adding varying concentrations of CuCl$_2$ (0.0005$\mu$M-100$\mu$M)/0.168$\mu$M Cu,Zn SOD.
Absorbance at 450nm

Cu(II)/Myricetin ratio

3x10^-6 6x10^-6 0.1 0.3 0.5 0.7 0.9 1.1
well as copper alone, we have carried out the experiment in which Cu,Zn SOD was incubated with varying concentrations of Cu (II) (0-2000 µM) alone as well as in combination with 1mM myricetin. The results are shown in Table 13. As evident from the table, there was a drastic increase in the Cu,Zn SOD activity as a function of increasing concentration of copper alone, when the values were calculated by considering the Cu,Zn SOD as a base control (100%). When the same experiment was repeated in presence of 1mM myricetin along with the increasing concentrations of Cu(II), a gradual decrease in the Cu,Zn SOD activity was observed when the values of Cu,Zn SOD in presence of the respective concentration of Cu(II) was taken as a base control. Based on several reports that flavonoids, in presence of Cu(II) generate hydroxyl radical (Cao, et al., 1997) it could be presumed from the above experiment, that gradual decrease in Cu,Zn SOD activity was due to some additional factor. In order to confirm whether myricetin, a flavonoid, in presence of Cu(II) leads to the formation of hydroxyl radical, we have carried out this interaction in presence of a hydroxyl radical scavenger, catalase. The results are shown in Figure 17. As evident from the figure, myricetin alone exhibited a marked inactivation of Cu,Zn SOD activity which was further enhanced in presence of 1mM CuCl2 and no restoration of enzyme activity has been achieved by adding increasing concentrations of catalase suggesting that there was no involvement of hydroxyl radical in presence of CuCl2 and myricetin. Since the uric acid is also considered as a radical scavenger (Ames, et al., 1981) of singlet oxygen, hydroxyl radical and other oxidants, we have further carried out the Cu,Zn SOD and myricetin interaction in presence of Cu(II) and uric acid. The results are shown in Figure 18. As evident from the figure, there was no restoration of Cu,Zn SOD activity in presence of uric acid.

The SDS-polyacrylamide gel electrophoresis of Cu,Zn SOD was carried out in presence of CuCl2 alone as well as in combination with 1mM myricetin. The results are shown in Figure 19. There was no change in the electrophoretic pattern of Cu,Zn SOD either treated with CuCl2 alone or in combination with myricetin suggesting that no fragmentation of Cu,Zn SOD has occurred even after its almost complete inactivation.
Table 13 Effect of varying concentrations of copper on Cu,Zn SOD activity in presence and absence of myricetin.

The reaction mixture containing 7.5μM Cu,Zn SOD was incubated with and without 1mM myricetin in presence of increasing concentrations of Cu(II) at 37°C for 3 hours. After the reaction period was over, the eppendorfs were centrifuge briefly and suitable volumes of the aliquots were used for measuring Cu,Zn SOD activity. The percent remaining activity was based on (A) by considering the value of Cu,Zn SOD control as 100% (B) values of individual Cu(II) concentration incubated with Cu,Zn SOD were taken as 100 percent.

<table>
<thead>
<tr>
<th>Concentration of Cu, Zn SOD (7.5μM)</th>
<th>Concentration of CuCl₂ (μM)</th>
<th>Concentration of myricetin (1mM)</th>
<th>Percent Remaining Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>+</td>
<td>20.6</td>
</tr>
<tr>
<td>+</td>
<td>100</td>
<td>-</td>
<td>134 (B) 100</td>
</tr>
<tr>
<td>+</td>
<td>100</td>
<td>+</td>
<td>35.5 (B) 26.4</td>
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<td>+</td>
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<td>140 (B) 100</td>
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<td>+</td>
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<td>31.7 (B) 19.6</td>
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<td>+</td>
<td>600</td>
<td>-</td>
<td>144 (B) 100</td>
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<td>16.9 (B) 9.2</td>
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<td>+</td>
<td>1000</td>
<td>-</td>
<td>134 (B) 100</td>
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<td>+</td>
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<td>+</td>
<td>6 (B) 4.4</td>
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<tr>
<td>+</td>
<td>2000</td>
<td>-</td>
<td>136 (B) 100</td>
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<tr>
<td>+</td>
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<td>+</td>
<td>7.6 (B) 5.5</td>
</tr>
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</table>
Figure 17. Effect of catalase on the activity of Cu,Zn SOD in presence of myricetin/Cu(II).

Reaction mixtures containing 7.5μM Cu,Zn SOD, 1mM myricetin and 1mM Cu(II) were incubated with increasing concentrations of catalase for 3 hours at 37°C. After the incubation period was over, the reaction samples were briefly centrifuged and appropriate volumes of the supernatant were used for the determination of Cu,Zn SOD activity.

1. Cu,Zn SOD control
2. Cu,Zn SOD treated with myricetin
3. Cu,Zn SOD treated with Cu(II) alone
4. Cu,Zn SOD treated with myricetin in presence of Cu(II)
5. Cu,Zn SOD treated with myricetin and Cu(II) in presence of 0.4μg catalase
6. Cu,Zn SOD treated with myricetin and Cu(II) in presence of 0.9μg catalase
7. Cu,Zn SOD treated with myricetin and Cu(II) in presence of 1.5μg catalase
8. Cu,Zn SOD treated with myricetin and Cu(II) in presence of 2.0μg catalase
Figure 18 Effect of uric acid on myricetin treated Cu,Zn SOD.

In a total reaction mixture of 67μl, 7.5μM Cu,Zn SOD was incubated with 1mM myricetin and Cu(II) in presence/absence of uric acid at 37°C for 3 hours. After incubation period was over, suitable aliquots were withdrawn and Cu,Zn SOD activity was determined.

1. Cu,Zn SOD control
2. Cu,Zn SOD treated with myricetin
3. Cu,Zn SOD treated with Cu(II) alone
4. Cu,Zn SOD treated with myricetin in presence of Cu(II)
5. Cu,Zn SOD treated with Cu(II) in presence of uric acid
6. Cu,Zn SOD treated with myricetin and Cu(II) in presence of uric acid
**Figure 19** SDS-Gel Electrophoretic pattern of Cu,Zn SOD treated with myricetin in presence and absence of CuCl$_2$.

In a total reaction mixture of 33µl, 7.5µM Cu,Zn SOD was treated with 1mM myricetin in presence and absence of 1mM CuCl$_2$ and incubated for 3 hours at 37°C. Cu,Zn SOD alone and in presence of 1mM CuCl$_2$ only was also incubated under similar conditions and after the incubation period, suitable aliquots (≈ 4µg protein) were withdrawn and analyzed by SDS gel electrophoretic pattern. The gel was stained by silver nitrate as described in the text.

a - Marker protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with myricetin

d - Cu,Zn SOD treated with Cu(II)

e - Cu,Zn SOD treated with myricetin in presence of Cu(II)
In order to confirm the possibility of the interaction of Cu(II) of Cu,Zn SOD with myricetin, an experiment was carried out in which the different concentrations of Cu(II) were incubated with a fixed concentration of myricetin prior to the incubation with Cu,Zn SOD. The results are shown in Figure 20. As shown in the figure, at 0.1mM myricetin there was 40% inactivation of the enzyme. When the Cu(II) was incubated with myricetin for 18 hours, the precipitate of Cu(II)-myricefin complex started appearing at a particular concentration of Cu(II). When the supernatant from these complexes were incubated with Cu,Zn SOD, there was a gradual decrease in the inactivation of Cu,Zn SOD suggesting that no free myricetin was available at maximum concentration of CuCl₂ used in this experiment resulting in almost complete restoration of Cu,Zn SOD activity.

**Interaction of Cu,Zn SOD with myricetin involving different metal ions**

From the studies conducted so far, it has been demonstrated that myricetin alone inactivates the Cu,Zn SOD activity, Cu(II) alone activate the Cu,Zn SOD while the same metal ion in combination with myricetin increases the percent inactivation of the enzyme. The role of other metal ions in such type of interactions is not known. We have performed an experiment, in which the Cu,Zn SOD was allowed to interact with myricetin in combination with Fe(III), Cd(II) and Mn(II). The results are shown in Figure 21. As shown in the figure, among the metal ions used for this study, iron was the one which maximally inactivated the Cu,Zn SOD activity while the other metal ions at the same concentration as well as under similar conditions partially inactivated the enzyme. The Cu,Zn SOD activity was completely lost in presence of myricetin and Fe(III) while the cadmium and manganese remained ineffective in presence of myricetin. The SDS polyacrylamide gel electrophoretic pattern of the samples of Cu,Zn SOD treated with myricetin in presence and absence of Fe(III) and Mn(II) is shown in Figure 22 and Figure 23 respectively. As evident from figure 22,1mM Fe (III) alone caused considerable fragmentation of Cu,Zn SOD while in presence of 1mM myricetin there was a complete fragmentation of the enzyme as suggested by the faint band in lane e.
In a total reaction volume of 300μl, 0.5mM myricetin was incubated with varying concentrations of CuCl₂ (0-1000μM) for 18 hours at 4°C. In the control set of myricetin, volume of CuCl₂ was replaced by 1.5mM potassium phosphate buffer pH 6.8. The samples were centrifuged briefly and equal volumes of the supernatant from each sample (~ 0.1mM myricetin in the control set) were incubated with 7.5μM Cu,Zn SOD for 3 hours at 37°C and Cu,Zn SOD activity was determined. The value of equal concentrations of Cu,Zn SOD without the supernatant was taken as 100% for calculating the percent remaining activity in each sample. These values represent the average of at least three independent experiments performed in duplicates.
The separate reaction mixtures containing 7.5 μM Cu,Zn SOD were incubated with different metal ions at a final concentration of 1 mM each in presence and absence of 1 mM myricetin. The incubation was carried out at 37°C for 3 hours. After the incubation period was over, activity of Cu,Zn SOD was determined in a suitable volume of aliquots.

A - 1. Cu,Zn SOD control  2. Cu,Zn SOD + 1 mM myricetin

B - 1. Cu,Zn SOD control  2. Cu,Zn SOD + MnCl₂  3. Cu,Zn SOD + MnCl₂ + myricetin

C - 1. Cu,Zn SOD control  2. Cu,Zn SOD + CdCl₂  3. Cu,Zn SOD + CdCl₂ + myricetin

D - 1. Cu,Zn SOD control  2. Cu,Zn SOD + CuCl₂  3. Cu,Zn SOD + CuCl₂ + myricetin

E - 1. Cu,Zn SOD control  2. Cu,Zn SOD + FeCl₃  3. Cu,Zn SOD + FeCl₃ + myricetin
Figure 22 SDS-Gel Electrophoretic pattern of Cu,Zn SOD treated with myricetin in presence and absence of Fe(III).

7.5μM Cu,Zn SOD was incubated with 1mM Fe(III) in absence and presence of 1mM myricetin for 3 hours at 37°C. After the incubation period, proper aliquots were loaded on 12.5% SDS-PAGE. Staining was done by silver nitrate as described in the text.

a - Marker protein
b Cu,Zn SOD control
c - Cu,Zn SOD treated with myricetin
d - Cu, Zn SOD treated with Fe(III) alone
e - Cu, Zn SOD treated with myricetin in presence of Fe(III)
From the results of Figure 23 it is suggested that there was no change in the electrophoretic pattern of Cu,Zn SOD either treated with MnCl₂ alone or in combination with myricetin.

In order to study the possible interaction of myricetin with Fe(III), an experiment was performed in which different concentrations of Fe(III) were incubated with a fixed concentration of myricetin prior to the incubation with Cu,Zn SOD. As shown in Figure 24, when Fe(III) was incubated with myricetin for 18 hours, and then supernatant (≈ 0.1mM myricetin in the control set) of each sample was incubated with Cu,Zn SOD, there was a gradual decrease in the percent inactivation of the enzyme indicating that almost all of the myricetin was used up in forming the complex with Fe(III) and free myricetin was not available to interact with Cu,Zn SOD and cause inactivation. The same protocol was also followed to determine the interaction of myricetin with another metal ion, Mn(II), in this experiment, a fixed concentration of myricetin (0.5mM) was incubated with varying concentrations of Mn(II) (0-1000µM) for 18 hours at 4°C. After the incubation period, the supernatants from these samples were incubated with Cu,Zn SOD and no restoration of Cu,Zn SOD activity was achieved (Figure 25). This suggests that unlike Cu(II) and Fe(III), Mn(II)-myricetin complex contributes in further inactivation of the Cu,Zn SOD.

Quercetin - Cu,Zn SOD

Quercetin is a flavonol present in vegetables, fruit skins, onions etc. It works as an antioxidant by scavenging the free radicals and hence prevents the damage caused by them. Quercetin offers a variety of potential therapeutic uses, primarily in the prevention and treatment of allergies, asthma, hayfever and hives. In order to study the interaction of Cu,Zn SOD with quercetin, we have carried out certain preliminary studies where, the enzyme (Cu,Zn SOD) was incubated with varying concentrations of quercetin in presence and absence of Cu(II) for different time intervals (3 hours and 18 hours) separately at 37°C. As shown in Figure 26, with increasing concentrations of quercetin, in the absence of Cu(II) there was an
Figure 23 SDS-Gel Electrophoretic pattern of Cu,Zn SOD treated with myricetin in presence and absence of Mn(II).

7.5 μM Cu,Zn SOD was treated with 1mM myricetin in absence and presence of Mn(II) at a final concentration of 1mM and incubated for 3 hours at 37°C. After the incubation period was over, samples were subject SDS polyacrylamide gel electrophoresis and staining was done by silver nitrate.

a - Marker protein
b - Control Cu,Zn SOD control
c - Cu,Zn SOD treated with myricetin
d - Cu,Zn SOD treated with Mn(II)
e - Cu,Zn SOD treated with myricetin in presence of Mn(II)
Figure 24 Formation of myricetin-Fe(III) complex and their interaction with Cu,Zn SOD.

In a total reaction volume of 300μl, 0.5mM myricetin was incubated with varying concentrations of FeCl₃ (0-1000μM) for 18 hours at 4°C. In the control set of myricetin, volume of FeCl₃ was replaced by 1.5mM potassium phosphate buffer pH 6.8. The samples were centrifuged briefly and equal volumes (≅ 0.1mM myricetin in the control set) were incubated with 7.5μM Cu,Zn SOD for 3 hours at 37°C and Cu,Zn SOD activity was determined. The value of equal concentrations of Cu,Zn SOD without the supernatant was taken as 100% for calculating the percent remaining activity in each sample. These values represent the average of at least three independent experiments performed in duplicates.
Figure 25 Formation of Myricetin-Mn(II) complex and their interaction with Cu,Zn SOD.

In a total reaction volume of 300μl, 0.5mM myricetin was incubated with varying concentrations of MnCl₂ (0-1000μM) for 18 hours at 4°C. In a control set of myricetin, volume of MnCl₂ was replaced by 1.5mM potassium phosphate buffer pH 6.8. The samples were centrifuged briefly and equal volumes of the supernatant from each sample (~0.1mM myricetin in the control set) were incubated with 7.5μM Cu,Zn SOD for 3 hours at 37°C and Cu,Zn SOD activity was determined. The value of equal concentrations of Cu,Zn SOD without supernatant was taken as 100% for calculating the percent remaining activity in each sample. These values represent the average of at least three independent experiments performed in duplicates.
In a reaction volume of 33μl, 7.5μM Cu,Zn SOD was treated with varying concentrations of quercetin (50-500μM) in presence and absence of 1mM CuCl₂ for 3 hours at 37°C. After the incubation period, proper aliquots were withdrawn for determining Cu,Zn SOD activity. Control Cu,Zn SOD (without quercetin and copper) was also incubated under similar conditions and considered to have 100% activity. Calculations of treated sets were compared with the control set.

- Cu,Zn SOD treated with quercetin in absence of 1mM Cu(II).
- Cu,Zn SOD treated with quercetin in presence of 1mM Cu(II).
activation of Cu,Zn SOD activity. Incubation of enzyme with quercetin in presence of Cu(II) resulted in a significant decrease in the activity of Cu,Zn SOD and that the decrease was time dependent (Figures 26 and 27).

SDS-gel electrophoretic pattern of the above samples incubated for 3 hours and 18 hours are shown in Figure 28 and Figure 29 respectively. As shown in figure 28, when the incubation of Cu,Zn SOD was done with 500 μM quercetin, in presence and absence of Cu(II) for 3 hours at 37°C, there was no degradation of the protein. Incubation of the above samples for 18 hours under same conditions caused complete fragmentation of Cu,Zn SOD treated with 500 μM quercetin in presence of Cu(II) as depicted by lane d (Figure 29).

*Spectral studies on quercetin- Cu,Zn SOD complex*

Interaction of quercetin with Cu,Zn SOD was also studied by the UV/visible absorption spectroscopy and fluorescence spectroscopy. Figure 30 shows an absorption spectra of untreated, Cu,Zn SOD and quercetin treated, dialyzed Cu,Zn SOD. As depicted from the figure, the untreated Cu,Zn SOD gives a major peak at 205 nm and a minor peak at 270 nm. A hypsochromic and hypochromic shift was observed in case of Cu,Zn SOD treated with quercetin as compared to that of the control untreated Cu,Zn SOD. Intrinsic fluorescence of the dialyzed treated and untreated Cu,Zn SOD is shown in Figure 31. As it is clear from the figure, the emission spectra obtained for untreated/native Cu,Zn SOD gives emission maximum at 292 nm, when excited at 280 nm. Treatment with quercetin caused a 1 nm blue shift of the emission maximum with a drastic increase in the intensity of fluorescence.

*Rutin - Cu,Zn SOD*

Studies on the interaction of rutin and Cu,Zn SOD were also conducted in order to find out whether rutin modulates the Cu,Zn SOD activity. The effect of increasing concentrations (50-500 μM) in presence and absence of copper for different
Figure 27 Interaction of Quercetin with Cu,Zn SOD in presence and absence of Cu(II)

The reaction mixtures containing 7.5μM Cu,Zn SOD were incubated with varying concentrations of quercetin (50-500μM) in presence and absence of Cu(II) for 18 hours at 37°C. After the incubation period suitable aliquots were withdrawn to determine the activity of Cu,Zn SOD, considering that control Cu,Zn SOD has 100 percent activity.

- Cu,Zn SOD treated with quercetin in absence of 1mM Cu(II).
- Cu,Zn SOD treated with quercetin in presence of 1mM Cu(II).
Figure 28 SDS-Polyacrylamide gel electrophoresis of Cu,Zn SOD samples treated with quercetin in presence and absence of Cu(II).

Reaction mixtures of 33μl, containing 7.5μM Cu,Zn SOD was treated with 500μM quercetin in presence and absence of 1mM CuCl₂ and incubated for 3 hours at 37°C. The aliquots (≈ 4μg) were withdrawn from each sample, after the incubation period the samples were subjected to 12.5% SDS PAGE. The gel was stained by silver staining method as described in the text.

a - Marker protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with quercetin

d - Cu,Zn SOD treated with quercetin in presence of Cu(II).
Figure 29 SDS-Polyacrylamide gel electrophoresis of Cu,Zn SOD treated with quercetin in presence and absence of Cu(II).

A total reaction volume of 33μl, containing 7.5μM Cu,Zn SOD was treated with 500μM quercetin in presence and absence of 1mM CuCl₂ was incubated for 18 hours at 37°C. As the incubation period was over, proper volumes from the reaction mixtures were withdrawn (≈ 4μg protein) and loaded on the gel (12.5% SDS-PAGE). Staining was done by silver nitrate.

a - Marker protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with quercetin

d - Cu,Zn SOD treated with quercetin in presence of Cu(II).
The reaction mixture containing 7.5μM Cu,Zn SOD and 500μM quercetin was incubated at 37°C for 3 hours. Control sets were also run in which only Cu,Zn SOD and quercetin was present at the same concentration and incubated under similar conditions. The total volume was compensated with 1.5mM potassium phosphate buffer, pH 6.8. After the incubation was over the reaction mixtures were extensively dialyzed against 1.5mM potassium phosphate buffer, pH 6.8. Proper aliquots (∼56μg/ml protein) from each reaction mixture was used for spectral analysis.

Cu,Zn SOD control (———)

Cu,Zn SOD treated with Quercetin (- - - - -)
Figure 31 Changes in the fluorescence spectra of Cu,Zn SOD after interaction with quercetin.

Reaction mixture containing 7.5μM Cu,Zn SOD was incubated with 500μM quercetin at 37°C for 3 hours. Control sets containing quercetin and Cu,Zn SOD alone at same concentrations were also incubated under similar conditions. Total volume in the control sets was compensated with 1.5mM potassium phosphate buffer, pH 6.8. After the incubation period was over, the reaction mixtures were extensively dialyzed against 1.5mM potassium phosphate buffer, pH 6.8. Proper aliquots (~56μg protein/ml) were withdrawn from each sample and used for taking the spectra. The excitation wavelength was 280nm and scanning wavelength range was 250nm-400nm.

1. Cu,Zn SOD control

2. Cu,Zn SOD treated with quercetin
durations at 37°C as shown in figures 32 and 33. There was an activation of Cu,Zn SOD activity in presence of rutin. While an insignificant inactivation was observed when Cu,Zn SOD was incubated with rutin in presence of Cu(II) for a period of 3 hours (Figure 32). The rutin induced inactivation of Cu,Zn SOD activity in presence of Cu(II) was significantly enhanced when incubation period was 18 hours (Figure 33).

The SDS polyacrylamide gel electrophoretic patterns of rutin treated Cu,Zn SOD under different conditions are shown in figures 34 and 35. No difference was found in the electrophoretic pattern of rutin treated Cu,Zn SOD in presence and absence of Cu(II) incubated for 3 hours and 18 hours at 37°C.

**Spectral studies on rutin- Cu,Zn SOD complex**

The interaction of Cu,Zn SOD and rutin was further confirmed by different spectral studies. Figure 36, shows the absorption spectra of untreated dialyzed Cu,Zn SOD control as well as rutin treated Cu,Zn SOD. A major peak at 205nm and a minor peak at 270nm was given by control Cu,Zn SOD while the rutin treated, Cu,Zn SOD shows a 7nm hypsochromic and hypochromic shift in the major peak.

The intrinsic fluorescence spectra of the above samples is shown in Figure 37. Control Cu,Zn SOD was characterized by the emission maximum at 294nm when excited at 280nm, treatment with 1mM rutin caused a 2nm blue shift in the emission spectrum and 6% decrease in the intensity of the fluorescence.

**Naringenin - Cu,Zn SOD**

Another flavonoid selected for these studies was naringenin abundantly present in tomatoes, grapes and citrus fruits. The Cu,Zn SOD was treated with increasing concentrations of naringenin in presence and absence of exogenous copper. When the reaction mixtures were incubated at 37°C for 3 hours, there was a dose
Figure 32 Interaction of Cu,Zn SOD with rutin in presence and absence of Cu(II).

In a total reaction volume of 33 μl, 7.5 μM Cu,Zn SOD was treated with different concentrations of rutin (50-500 μM) in presence and absence of Cu(II) for 3 hours at 37°C. Control Cu,Zn SOD (without rutin and copper) prepared in 1.5 mM potassium phosphate buffer, pH 6.8 was also incubated under similar conditions. After the incubation period, suitable volume of the aliquots were withdrawn to determine Cu,Zn SOD activity. Activity of control Cu,Zn SOD was considered as 100 percent.

- Cu,Zn SOD treated with rutin in absence of 1 mM Cu(II).
- Cu,Zn SOD treated with rutin in presence of 1 mM Cu(II).
Concentration of rutin (μM)
Figure 33 Effect of rutin on Cu,Zn SOD activity in presence and absence of Cu(II).

Reaction mixtures, containing 7.5 μM Cu,Zn SOD and varying concentrations of rutin (50-500 μM) were incubated at 37°C for 18 hours. After the incubation was over, suitable volume of the aliquots were withdrawn and Cu,Zn SOD activity was determined.

- Cu,Zn SOD treated with rutin in absence of 1mM Cu(II).
- Cu,Zn SOD treated with rutin in presence of 1mM Cu(II).
Figure 34 SDS-polyacrylamide gel electrophoresis of Cu,Zn SOD treated with rutin in presence and absence of Cu(II).

7.5μM Cu,Zn SOD was treated with 500μM rutin in presence and absence of 1mM CuCl₂ for 3 hours at 37°C. After the incubation period, proper volume of the aliquots were withdrawn (~4μg protein) and loaded on the SDS-PAGE. The staining was done by silver nitrate as described in the text.

a - Marker protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with rutin alone

d - Cu,Zn SOD treated with rutin in presence of Cu(II)
Figure 35 SDS-polyacrylamide gel electrophoretic pattern of rutin treated Cu,Zn SOD in presence and absence of Cu(II).

A total reaction volume of 33μl, containing 7.5μM Cu,Zn SOD was treated with rutin (500μM rutin) in presence and absence of 1mM Cu(II) and incubated for 18 hours at 37°C. After the incubation period was over suitable aliquots were withdrawn (~ 4μg protein) and subjected to SDS-PAGE, finally the gel was stained by silver nitrate as explained in the text.

a - Marker protein

b - Cu,Zn SOD control

c - Cu,Zn SOD treated with rutin alone

d - Cu,Zn SOD treated with rutin in presence of Cu(II)
Figure 36 Absorption spectra of Cu,Zn SOD treated with rutin.

The reaction mixture containing 500µM rutin and 7.5µM Cu,Zn SOD was incubated at 37°C for 3 hrs. The control sets containing 1mM rutin and 7.5µM Cu,Zn SOD were prepared in 1.5mM potassium phosphate buffer pH 6.8 and incubated under similar conditions. After the incubation period the reaction mixtures were dialyzed extensively against 1.5mM potassium phosphate buffer pH 6.8 and proper aliquots from the dialyzed samples equivalent to (56µg/ml) protein were used for taking the spectra.

Cu,Zn SOD control(———)

Cu,Zn SOD treated with rutin (……..)
Figure 37 Fluorescence spectral analysis of interaction of Cu,Zn SOD with rutin.

Total reaction volume of 400µl, containing 7.5µl Cu,Zn SOD and 500µM rutin was incubated at 37°C for 3 hours. Control sets containing 7.5µM Cu,Zn SOD alone and 500µM rutin alone, prepared in 1.5mM potassium phosphate buffer pH 6.8 were also incubated under similar conditions. After the incubation time was over, the reaction mixtures were dialyzed extensively against 1.5mM potassium phosphate buffer, pH 6.8. Suitable volumes of dialyzates were withdrawn (≈ 56µg protein/ml) and used for spectral analysis.

1. Cu,Zn SOD control

2. Cu,Zn SOD treated with rutin
dependent decrease in the activity of Cu,Zn SOD in presence of naringenin alone, while a further decrease in the activity was observed in presence of naringenin and Cu(II) (Figure 38). Almost a similar pattern was observed when the reaction mixtures were incubated for a longer durations. As shown in Figure 39, when Cu,Zn SOD was incubated with naringenin alone for 18 hours at 37°C, a dose dependent decrease in the enzyme activity was observed which was further decreased when Cu,Zn SOD was treated with naringenin in presence of 1mM CuCl₂.

The reaction mixtures containing Cu,Zn SOD treated with 500μM naringenin in presence and absence of 1mM CuCl₂ were incubated at 37°C for 3 hours and 18 hours separately and subjected to SDS polyacrylamide gel electrophoresis. Figure 40, shows the electrophoretic pattern of the samples incubated at 37°C for 3 hours. A decrease in the band intensity of Cu,Zn SOD treated with naringenin in presence of 1mM Cu(II) was observed (lane d). Figure 41, shows the gel pattern of Cu,Zn SOD samples treated under identical conditions for the duration of 18 hours. Almost similar band pattern was observed in all samples (lane b-d).

**Spectral studies on naringenin- Cu,Zn SOD complex**

The absorption spectra of dialyzed samples of untreated and naringenin treated Cu,Zn SOD is given in Figure 42. As shown in the figure, the major and minor peaks of control Cu,Zn SOD were observed at 205nm and 270nm respectively. In case of naringenin treated Cu,Zn SOD, there was again a hypsochromic shift both in major and minor peak while hypochromic shift was seen in major peak only. The fluorescence emission spectra of the above samples are given in Figure 43. The emission of control Cu,Zn SOD shows λ_max at 295nm when excited at 280nm while the naringenin treated Cu,Zn SOD showed a blue shift of 5nm and an increase in the fluorescence intensity.
Cu,Zn SOD at a final concentration of 7.5μM was incubated with varying concentrations of naringenin (50-500μM) in presence and absence of 1mM Cu(II) for 3 hours at 37°C. After the incubation was over, suitable volume of the aliquots were withdrawn and Cu,Zn SOD activity was determined. Similar concentration of Cu,Zn SOD prepared in buffer only (1.5mM potassium phosphate buffer pH 6.8) was also incubated under similar conditions.

(●) Cu,Zn SOD treated with naringenin alone.

(■) Cu,Zn SOD treated with naringenin in presence of CuCl₂
Figure 39 Effect of naringenin on Cu,Zn SOD activity in presence and absence of Cu(II).

A total reaction volume of 33µl, containing 7.5µM Cu,Zn SOD was treated with varying concentrations of naringenin (50-500µM) in presence and absence of 1mM CuCl₂ and incubated for 18 hours at 37°C. An enzyme control, containing 7.5µM Cu,Zn SOD in 1.5mM potassium phosphate buffer, pH 6.8 only was also incubated under similar conditions. After the incubation period was over, suitable aliquots were withdrawn and Cu,Zn SOD activity was determined.

○ Cu,Zn SOD treated with naringenin alone

■ Cu,Zn SOD treated with naringenin in presence of Cu(II)
Figure 40 SDS-Gel electrophoretic pattern of Cu,Zn SOD incubated with naringenin in presence and absence of Cu(II).

7.5μM Cu,Zn SOD, treated with 500μM naringenin in presence and absence of 1mM CuCl₂ was incubated for 3 hrs at 37°C. Proper volumes of the aliquots were withdrawn (≈ 4μg protein) and analyzed on SDS polyacrylamide gel.

a - Marker protein
b - Cu,Zn SOD Control
c - Cu,Zn SOD treated with naringenin
d - Cu, Zn SOD treated with naringenin in presence of Cu(II)
Figure 41 SDS-Gel electrophoretic pattern of Cu,Zn SOD incubated with naringenin in presence and absence of Cu(II).

In a total reaction volume of 33μl, 7.5μM Cu,Zn SOD was incubated with 500μM naringenin in presence and absence of 1mM CuCl₂ for 18 hours at 37°C. Suitable volumes (~ 4μg protein) from each sample were analyzed on SDS Polyacrylamide gel.

a - Marker protein
b - Cu,Zn SOD control
c - Cu,Zn SOD treated with naringenin
d - Cu,Zn SOD treated with naringenin and Cu(II)
Figure 42 UV/Visible spectra of Cu,Zn SOD treated with Naringenin

Total reaction volume of 400μl, containing 7.5μM Cu,Zn SOD and 500μM naringenin was incubated at 37°C for 3 hours. The control set of Cu,Zn SOD alone, was also incubated under similar conditions. The reaction volume, in the control set was compensated by the 1.5mM potassium phosphate buffer, pH 6.8. After the incubation period was over, the reaction mixtures were extensively dialyzed against 1.5mM potassium phosphate buffer, pH 6.8. Suitable volumes of the reaction mixtures were withdrawn (~ 56μg protein per ml) and spectra was recorded.

Cu,Zn SOD control. (—)

Cu,Zn SOD treated with naringenin (-----)
Figure 43 Fluorescence emission spectra of Cu,Zn SOD treated with naringenin.

Reaction mixtures containing 7.5μM Cu,Zn SOD was incubated with 500μM naringenin at 37°C for 3 hours control set of naringenin and Cu,Zn SOD alone, were also incubated under similar conditions. The volume of the control sets was compensated by 1.5mM potassium phosphate buffer, pH 6.8. After the incubation period was over, the reaction sets were extensively dialyzed against 1.5mM potassium phosphate buffer, pH 6.8. Proper aliquots (~ 56μg protein/ml) were withdrawn from each sample and used for spectral study. The excitation wavelength was 280nm whereas scanning of the spectra was done at 250-400nm.

1. Cu,Zn SOD control
2. Cu,Zn SOD treated with naringenin
In Vivo Studies

The in vivo studies were also carried out where myricetin and quercetin were injected into the mice and the liver homogenates were used for measuring Cu,Zn SOD activity. The results are shown in figures 44-47. As evident from the figures there was a 40% decrease in Cu,Zn SOD activity in myricetin treated mice (Figure 44), while slight activation was observed in case of samples obtained from quercetin treated mice (Figure 46), when compared with control samples. When the Cu,Zn SOD activity staining was done after native polyacrylamide gel electrophoresis, the above samples, a corelation with the activity pattern was obtained.
2. Myricetin treated mice liver homogenate.

1. Control mice liver homogenate

Cu/Zn SOD activity.

Liver tissues were perfused and the homogenate was used for determining
injected with equal volume of ethanol. After 3 hours mice were sacrificed
myricetin dissolved in 0.5 ml of ethanol while the control groups were
Treated groups of mice were injected intraperitoneally with 0.5 mg

Figure 4: Effect of myricetin on mice liver Cu/Zn Superoxide dismutase.
Cu, Zn SOD activity (Units/mg protein)

Liver homogenates
Figure 45 Activity staining of myricetin treated Cu,Zn SOD from mice liver homogenate.

Control and myricetin treated mice liver homogenates were electrophoresed on 7.5% native polyacrylamide gel and the activity was stained as described in the text.

a- Control Cu,Zn SOD

b- Control mice liver homogenate

c- Control mice liver homogenate

d- myricetin treated mice liver homogenate

e- myricetin treated mice liver homogenate
Figure 46 Effect of quercetin on mice liver Cu,Zn Superoxide dismutase.

Treated groups of mice were injected intraperitoneally with 0.3 mg quercetin dissolved in 0.5 ml of ethanol while the control groups were injected with equal volume of ethanol. Mice were sacrificed after 3 hours of injection, liver tissues were perfused and its homogenate was used for determining Cu,Zn SOD activity.

1. Control mice liver homogenate

2. Quercetin treated mice liver homogenate.
Liver homogenates

Cu, Zn SOD activity (Units/mg protein)
Figure 47 Activity staining of Cu,Zn SOD from mice liver treated with quercetin

Control and quercetin treated mice liver homogenates were electrophoresed on native 7.5% polyacrylamide gel and the activity was stained by the procedure described in the text.

a- Control Cu,Zn SOD
b- Control mice liver homogenate
c- Control mice liver homogenate
d- Quercetin treated mice liver homogenate
e- Quercetin treated mice liver homogenate