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# Naringin and naringenin inhibit nitrite-induced methemoglobin formation

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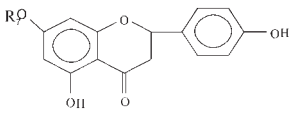
Naringin and naringenin protect hemoglobin from nitrite-induced oxidation to methemoglobin. The protection is not observed when naringin and naringenin are added after the autocatalytic stage of the oxidation of hemoglobin by nitrite. The ability of naringin and naringenin to scavenge oxygen free radicals may be responsible for the action because superoxide, hydroxyl and other free radicals are implicated in promoting the autocatalytic stage of oxidation of hemoglobin by nitrite. Both compounds showed less ability to protect intact erythrocytes suggesting that they may not cross the erythrocyte membrane in sufficient amounts. Naringenin was more effective than naringin, probably because of the extra phenolic group in the aglycone.

## 1. Introduction

Nitrite is produced when *Nitrosomonas* sp. bacteria oxidize ammonia from fish and decomposing organic matter [1]. Excess nitrite in water possess an ecological threat to the aquafauna. High nitrite levels lead to increased active transport across gills into the fish's blood stream where it oxidizes hemoglobin to methemoglobin [2] that cannot transport oxygen. This leads to asphyxia, and respiratory diseases in fish [2]. Several antioxidants have been shown to prevent nitrite-induced oxidation of hemoglobin to methemoglobin [3–5] and may therefore play a role in protecting aquafauna. The formation of methemoglobin occurs in two stages. The first is a slow stage and the second is a rapid autocatalytic stage [3]. Several oxygen free radicals such as superoxide anion, hydrogen peroxide and nitrogen dioxide have been implicated in the autocatalytic stage [3]. Free radical scavengers and antioxidants such as superoxide dismutase, uric acid, ascorbic acid and glutathione are reported to protect hemoglobin against nitrite-induced oxidation [4].




Naringin ($R_7$ – Rhamnoglucosyl); Naringenin ($R_7$ – H)

Naringin and its aglycone naringenin are the flavanones found in grapefruit [6] and related *Citrus* species. Both naringin and naringenin are potent scavengers of reactive oxygen species [7, 8]. Since oxygen free radicals are involved in the nitrite-induced oxidation of hemoglobin, we thought it appropriate to investigate the ability of naringin and naringenin to protect hemoglobin against nitrite-induced oxidation.

## 2. Investigations, results and discussion

Nitrite causes a rapid oxidation of hemoglobin to methemoglobin. In the presence of naringin and naringenin, the oxidation process was delayed in a dose-dependent manner (Table 1). The time required to convert 50% of the available hemoglobin to methemoglobin was 18.1 min in control, whereas with 0.02 mM of naringin and naringenin the time increased to 28.1 and 85.9 min, respectively. However, curcumin (0.02 mM) showed better activity. Fig. 1 and 2 describe the effect of naringin (0.02 mM) and naringenin (0.02 mM) on the time-course of nitrite-induced oxidation of hemoglobin. Without the test compounds, the time-course of oxidation shows a characteristic pattern of slow initial transformation followed by a rapid autocatalytic process. When test compounds were added along with nitrite, i.e. at 0 min, the formation of

**Table 1: Inhibition of nitrite-induced methemoglobin formation in hemolysate by naringin and naringenin**

| Sample conc. (mM) | Time to form 50% methemoglobin[a] (min ± S.E.) |
|---|---|
| Control | 18.1 ± 1.8 |
| Curcumin | |
| 0.02 | 116.5 ± 5.8[b] |
| Naringin | |
| 0.001 | 16.7 ± 2.0[c] |
| 0.002 | 21.4 ± 2.4[c] |
| 0.02 | 28.1 ± 3.6[c] |
| Naringenin | |
| 0.001 | 27.3 ± 1.3[c] |
| 0.002 | 47.4 ± 4.3[c] |
| 0.02 | 85.9 ± 7.2[b] |

[a] All the values are mean ± standard error (S.E.), n = 3
[b] <0.01 compared to control
[c] <0.01 compared to curcumin

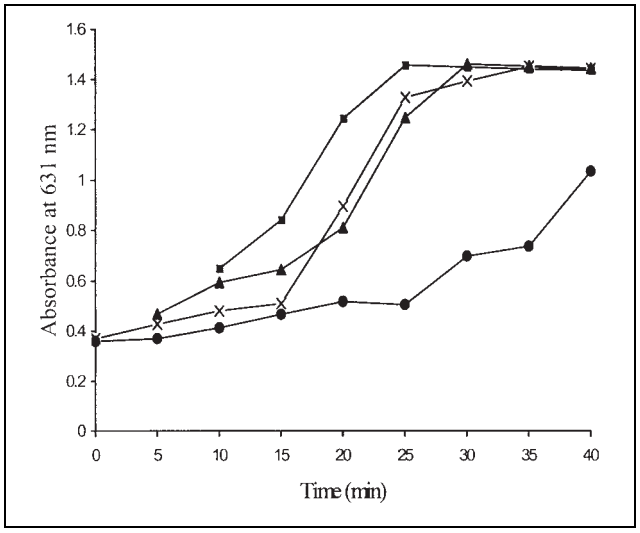



Fig. 1: Effect of naringin (0.02 mM) on time course of methemoglobin formation at 0 min (●), 5 min (▲), and 10 min (■); control (×)

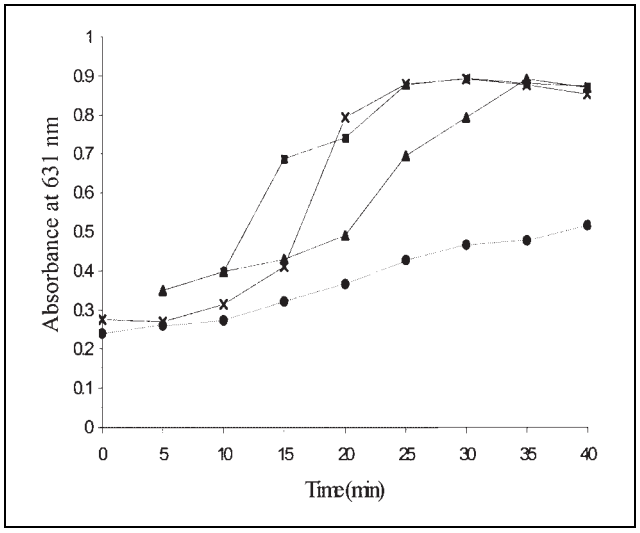



Fig. 2: Effect of naringenin (0.02 mM) on time course of methemoglobin formation at 0 min (●), 5 min (▲), and 10 min (■); control (×)

methemoglobin was inhibited, but the extent of inhibition was more for naringenin. Addition of naringin, 5 min after nitrite did not result in significant protection, where as naringenin showed appreciable protection when added 5 min after nitrite. However, when naringin and naringenin were added at the end of autocatalytic stage, i.e. at 10 min, no protection was observed. Both naringin (2 mM) and naringenin (2 mM) were able to inhibit the formation of methemoglobin in intact erythrocytes (Table 2), but to a very low extent as compared to curcumin.

The present study shows that naringenin and naringin are capable of protecting hemoglobin (in hemolysate) from nitrite-induced oxidation in the pre-autocatalytic stage. This trend was similar to what has been observed with other antioxidants such as ascorbic acid, uric acid, 3-ribosyl uric acid and glutathione [4]. The fact that naringenin and naringin are potent scavengers of oxygen free radicals [7, 8] suggests that the protective action may be by scavenging free radicals generated during the oxidation. Direct interaction with nitrite as a reason for protection is ruled out because both the test compounds show protection at a concentration as low as 0.001 mM, where as concentration of nitrite used is 0.6 mM. The inability of naringin and naringenin to protect intact erythrocytes as efficiently as curcumin suggests that they may not cross the membrane of erythrocytes as effectively as curcumin. Naringenin was more effective than naringin, suggesting that the extra phenolic group in the aglycone is important for activity. This is consistent with an earlier study [9], which states that the flavonoid aglycones were more potent in their antiperoxidative action than their corresponding glycosides.

**Table 2: Inhibition of nitrite-induced methemoglobin formation in erythrocytes by naringin and naringenin**

| Sample conc. (mM) | % Inhibition + S.E.[a] |
|---|---|
| Curcumin | |
| 0.4 | 48.4 ± 10.8 |
| 0.02 | 13.5 ± 4.8 |
| Naringin | |
| 2.0 | 11.5 ± 5.1 |
| 0.4 | 4.8 ± 4.2 |
| 0.02 | NA |
| Naringenin | |
| 2.0 | 17.5 ± 4.6 |
| 0.4 | 8.9 ± 3.3 |
| 0.02 | NA |

[a] Percent inhibition was calculated compared to control. All the values are mean ± standard error (S.E.); n = 3; (NA) Not active

## 3. Experimental

Curcumin, naringin and naringenin were obtained from Sigma Chemicals Co. and all other chemicals are of analytical grade. Human blood, collected into acid-citrate-dextrose, was from the blood bank of Kasturba Hospital, Manipal, India.

### *3.1. Inhibition of methemoglobin formation in hemolysate*

Blood samples were centrifuged (2500 × g, 20 min) to remove plasma and the buffy coat of white cells. Erythrocytes thus obtained were washed three times with phosphate-buffered saline and were lysed by suspending them in 20 volumes of 20 mM phosphate buffer, pH 7.4. The hemolysate was then centrifuged at 25000 × g for 60 min to remove the membranes, and then diluted to give 150 μM concentration of oxyhemoglobin. The reaction was initiated by the addition of sodium nitrite (final conc. 0.6 mM) to the solution of hemolysate and the formation of methemoglobin was measured by monitoring absorbance at 631 nm [10] using a Shimadzu Graphicord UV 240 spectrophotometer. The test compounds were added before or at various time intervals after the addition of nitrite. Control experiments were conducted without the test compounds and all experiments were done in triplicate. Results were compared to that of standard drug, curcumin.

### *3.2. Inhibition of methemoglobin formation in intact erythrocytes*

Erythrocytes washed in phosphated buffer saline, were incubated with the test compounds for 30 min followed by addition of sodium nitrite (final conc. 1.8 mM) for further 120 min. The suspension was centrifuged at 2500 × g for 20 min to remove excess test compounds and nitrite. The cells were washed thrice with phosphate-buffered saline and lysed with 20 mM phosphate buffer, pH 7.4. The lysate was then centrifuged at 25000 × g for 60 min and the amount of methemoglobin in the supernatant was measured as described above. The extent of methemoglobin formation by nitrite was calculated after taking into consideration the amount of methemoglobin already present in the erythrocytes before addition of nitrite. All experiments were done in triplicate.

### *3.3. Statistical evaluation*

Statistical evaluation of the data was done by one way ANOVA (Graph PAD Instar Software). A value of $p < 0.05$ was considered to be significant.

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