CHAPTER III
MATERIAL AND METHODS

1. Material

1.1 Chemicals

Curcuminoid capsules were supported by the government pharmaceutical organization. The following chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A.: brij 35 solution 30 %, drabkin's reagent, 5, 5'- dithio-bis, 2-nitrobenzoic acid (DTNB), glutathione reductase, reduced glutathione, oxidized glutathione, hemoglobin human, nicotinamide adenine · dinucleotide phosphate reduced form, nitroblue tetrazolium, superoxide dismutase, cumene hydroperoxide and 1-methyl-2-vinylpyridinium trifluoromethane sulfonate, trichoroacetic acid, thiobarbituric acid, xanthine disodium, xanthine Oxidase. All other reagents were analytical grade and obtained from commercial sources.

1.2 Instruments

The following instruments were major equipment used in this study:

1. Multiple microplate reader (Wallac model 1420)
2. Spectrophotometer (Jasco model UVDEC 650)
3. Spectrofluorometer (Jasco model FP-777)
4. Refrigerated centrifuge (Beckman coulter model Allegra X-12R)

2. Method

2.1 Subjects

Thirty-five patients with Leber's hereditary ophtic nuropathy were participated in this study. This is a randomized, double blind, placebo-control study. The protocol was reviewed and approved by the Ethical Committee on research involving human subject Faculty of Medicine, Siriraj Hospital, Mahidol University. All patients have mitochondrial DNA mutation at 11778 with symptom of visual impairment and have a special eye examination. Their liver and renal functions were normal. They were asked not to take any medication for their vision at least 1 month before the beginning

of the study, for example, coenzyme Q10, vitamin B, vitamin C, and vitamin E. The patients were divided in to 2 groups, treatment group and placebo group. Patients were treated with curcuminoid extracts capsule 250 mg or placebo capsule twice a day for 12 months. The antioxidant enzymes were monitor at 0, 3, 6 and 12 month.

2.2 Blood Samples and erythrolysates (Delalla and Gofman, 1954)

Ten ml of venous peripheral blood was collected into EDTA vacutainer tubes. The plasma was separated by centrifugation at 1200 g for 15 min. Plasma was collected for detection of malondialdeyde level (MDA). Then, the buffy coat was discharged and the remaining erythrocytes were washed and centrifuged repeatedly three times with 0.9% normal saline (1,200 g at 4°C for 15 min). Finally, a known volume of erythrocytes was lysated with equal volume of cold pure water.

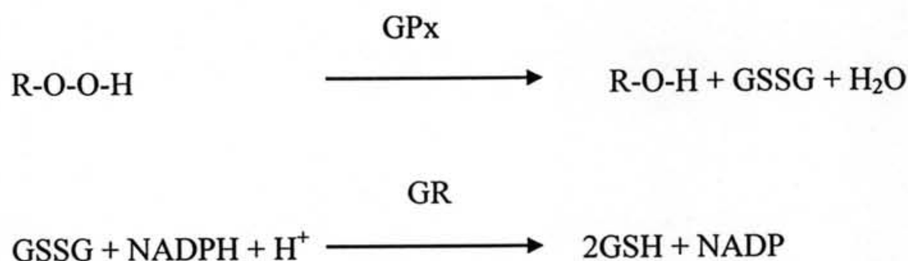
2.3 Determination of plasma MDA levels (Asakawa and Matsushita, 1980)

One ml of diluted plasma or standard solution was mixed with 50 μl of 100 μM butylated hydroxytoluene (BHT) to inhibit lipid peroxidation occurring during the process, then 1.0 ml of 10% trichloroacetic acid was added and mixed for 1 min. A 0.5 ml of 5 mM EDTA, 0.5 ml of 8% sodium dodecyl sulfate and 1.5 ml of 0.6% thiobarbituric acid, were added and vortex, respectively. The mixture reaction was boiled in water bath at 95 °C for 1 hr. After cooling, 4 ml of butanol was added. The mixture was vigorously vortex for 1 min then centrifuged at 1,500 g for 10 min. Fluorescence of the butanol extracts were measured at an excitation wavelength of 515 nm and emission wavelength of 553 nm.

2.4 Determination of Glutathione peroxidase activity (Paglia and Valentine, 1967)

Glutathione peroxidase (GPx) activities were measured by modification the method of Paglio and Valentine. The formation of GSSG catalyzed by GPx is couple to the recycling of GSSG back to GSH using glutathione reductase. NADPH is oxidized to $NADP^+$. The change in A340 due to NADPH oxidation was monitored and was indicative of GPx activity. The reaction mixture consisted of 5 mM Ethylene diamine tetraacetic acid-Na salt (EDTA), 0.1 M glutathione (GSH), 10 unit/ml glutathione reductase (GSH-Rd), Tris-HCl buffer pH 8.0. The hemolysate was added

to the mixture and allowed to incubate for 5 min at 37°C. The enzyme reaction was initiated by the addition of 7 mM cumene hydroperoxide that was used as the peroxide substrate (ROOH). The general reaction can be described as follows:



The rate of decrease in the absorbance at 340 nm was measured. Erythrocyte GPx activities were expressed as U/g Hb.

2.5 Determination of Catalase activity (Pippenger et al., 1998)

The determination of catalase activities were performed by a spectrophotometric assay based on the catalyzed decomposition of H_2O_2. To determine the catalase activity, 10 mM H_2O_2 in 50 mM potassium phosphate buffer should have H_2O_2 concentration 10 + 0.5 mM. Using the following formula:

$$H_2O_2 \text{ conc (mM/L)} = 21.81 \times (A240) - 0.36$$

Then add 3 ml of 10 mM H_2O_2 in 50 mM potassium phosphate buffer to cuvet and pre-warm at 25°C for 5 min. After that add 20 µl of hemolysate and record the change in absorbance at 240 nm between 30 and 210 sec. Catalase activities are express as KU/g Hb and calculate from

$$\text{CAT (KU/g Hb)} = \frac{A240}{0.43 \times 0.02 \times (\text{g/ml Hb})}$$

One unit of activity is defined arbitrarity as the amount of enzyme, which induces a change in A240 of 0.43 during the 3 min incubation.

2.6 Determination of total glutathione (Anderson, 1985)

Determination of total glutathione was based on the GSH recycling method. In this reaction GSH reacts with 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) to form disulfide GSTNB and the yellow colored compound, 5- thio-2-nitrobenzoic acid (TNB). The disulfide product (GSTNB) is then reduced by glutathione reductase in the presence of NADPH, recycling GSH back into the reaction following the scheme.

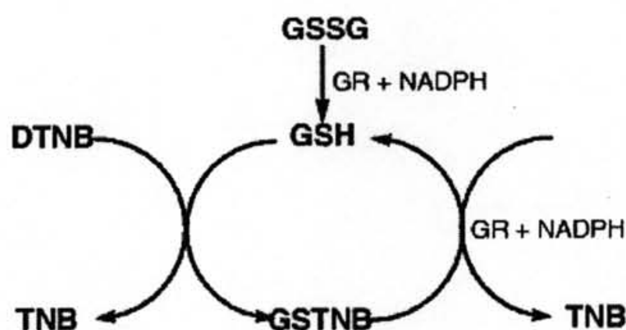


Figure 3.1 The GSH recycling method.

The rate of TNB formation was followed at 405 nm and was proportional to the concentration of GSH in the sample. Preparation of sample by add 0.5 ml of 4% sulfosalicylic into 0.5 ml of hemolysate, then centrifuge 12,000 rpm for 15 min at 4°C Diluted sample(20 µl), were transferred to 96-well microplate. Then add 80 µl of 0.01 sodium phosphate buffer with 1 mM EDTA pH 7.5. Subsequently, 100 µl of reaction mixture, which contained 1 mM of DTNB, 0.5 mM of NADPH, 1 iu of GSH reductase dissolved in 0.01 M of Sodium Phosphate buffer containing 1 mM of EDTA pH 7.5 (freshly prepare) were added immediately after addition of the reagent, color development was recorded at 405 nm for 4 min.

2.7 Determination of oxidized glutathione (GSSG) (Anderson, 1985)

GSSG was determined with the same method of total glutathione, but free GSH must be masked first by 1-methyl-2-vinylpyridinium trifluoromethane sulfonate (M2VP), after addition 0.5 ml of 4% sulfosalisylic acid the hemolysate were diluted

2-fold using 15 mM of M2VP in 1mM HCl (marking for GSH), following incubation at room temperature for 2 min then determine GSSG same as method of determine of total GSH.

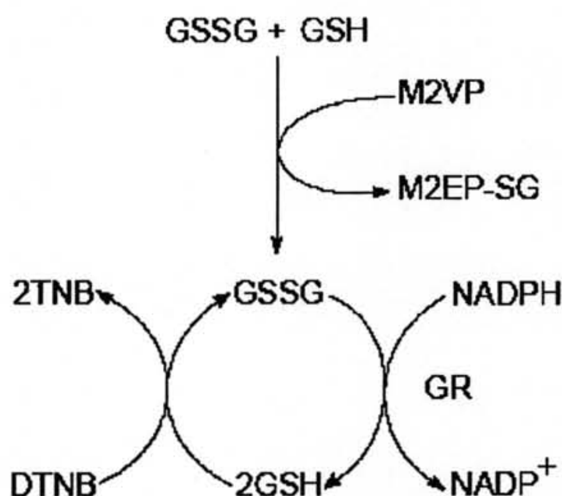
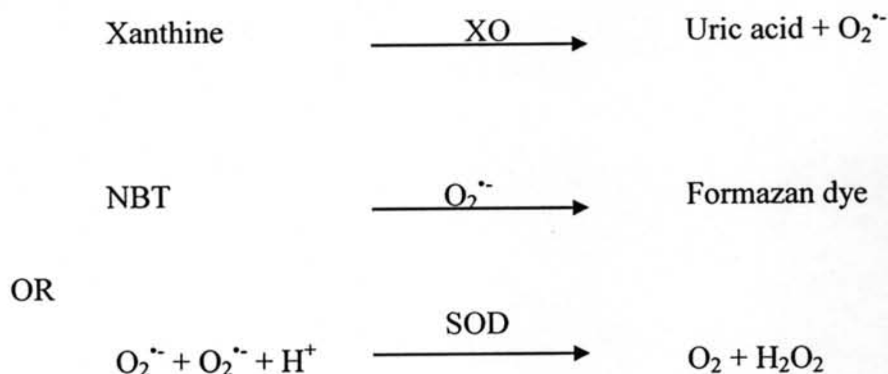


Figure 3.2 The M2VP maker in GSSG determination method

2.8 Determination of Superoxide Dismutases (Oberley and Spitz, 1984)

Superoxide dismutase was measured by using a modified method of Oberley and Spitz. This method employs xanthine and xanthine oxidase (XO) to generate superoxide radicals which react with nitroblue tetrazolium (NBT) to form a formazan dye. The role of NBT reduction was used as the reference rate. When increasing of SOD activity was added to the system, the rate of NBT reduction was inhibited. One unit of SOD is that which cause a 50% inhibition of the rate of reduction of NBT. The assay is diluted hemolysate by 0.01 phosphate buffer and aliquot 5 μl transfer to 96-well microplate. Subsequently, 170 μl of mixed substrate which contained 0.05 M of xanthine, 0.025 M of NBT, dissolved in 50 mM sodium carbonate buffer with EDTA pH 10.2, mixed well, then add 25 μl of 60 unit/L of xanthine oxidase. Immediately record a change of absorbance at 560 nm every 30 sec for 4 min.



2.9 Determination of Hemoglobin (Drabkin and Austin, 1935)

This procedure is based on the oxidation of hemoglobin and its derivatives to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin which has maximum absorption at 540 nm. To prepare the Drabkin's Solution, reconstitute one vial of the Drabkin's Reagent with 1,000 ml of water. Then add 0.5 ml of the 30% Brij 35 solution, mixed well. Add 5.0 ml of the Drabkin's solution in test tube, add 20 µl of hemolysate, Mix well and allow standing for at least 15 min at room temperature. Then record absorbance at 540 nm. Determine the total hemoglobin concentration (mg/ml) from the calibration curve of cyanmethemoglobin standard solution.

3. Statistical Analysis

The data were analyzed by using SPSS version 13.0 and express as mean ± SE. Mann-Whitney-U Test was used to test for comparison between groups who have curcuminoid extracts capsule and placebo. The correlation analyses were assessed by Spearman correlation. *P value* < 0.05 was considered as significant difference.