Experimental

8.1. Materials

Commercially available reagents (1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), reduced β-nicotinamide adenine dinucleotide (NADH), 5-methylphenazium methyl sulfate (PMS), nitro blue tetrazolium salt (NBT), triton X-100, standard radical scavengers; propyl gallate, 3-*t*-butyl-4-hydroxyanisole, 7,8-dihydroxy flavone). Solvents; dimethylsulfoxide, carbon tetracholride and ethanol, and the enzyme xanthine oxidase (butter milk), its substrate, xanthine and inhibitor allopurinol were all purchased from Sigma Chemical Co. or Fluka. 4-[3-(4-Iodophenyl)-2-(4-nitropheyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan) and ficoll paque from Pharmacia Biotech (Switzerland). Water used for buffer preparation was deionized by Simplicity Water Purification System (Millipore). Cytotoxicity and antiradical studies against 1,1-diphenyl-2-picrylhydrazyl radicals were carried out at 37° C, while the enzymatic reactions and superoxide scavenging studies were carried out at 28° C. All studies were performed in 96-well microtitre plates using Spectra Max-340 and Spectra Max-384 spectrophotometers (Molecular Devices, USA).

8.2. *In Vitro* Assays

A number of assays were used for measuring the antioxidant potential of the test samples. Depending on the mechanism, methods for the evaluation of antioxidant activities of the test compounds were divided into two categories:

1. Methods determining the ability of test compounds to donate an electron to any electron acceptor.

2. Methods which determine the ability of a sample to inhibit the enzymes, which produce reactive oxygen species.

8.2.1. DPPH Radical Scavenging Assay

Free radical scavenging abilities of the test compounds were determined by measuring the change in absorbance of DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) at 515 nm by the spectrophotometric method described by S. K. Lee (Lee *et al.*, 1998).

8.2.1.1. Principle

DPPH$^•$ (1,1-diphenyl-2-picrylhydrazyl radical) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. The radical scavenging potential of the compounds is determined by measuring the decrease in absorbance due to DPPH$^•$ at 517 nm, representing the formation of its reduced form, 1,1-diphenyl-2-picrylhydrazine (DPPH), which is yellow in color. Because of the odd electron, the purple colored ethanolic solution shows a strong absorption band at 517 nm (Blois, 1958). The mechanism of reaction is presented in Figure 8.1.

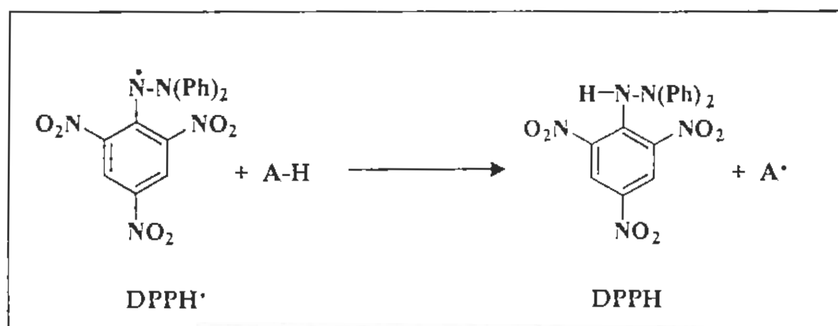

Figure 8.1: Mechanism of DPPH$^•$ with an antioxidant having a transferable hydrogen radical.

8.2.1.2. Method

The reaction mixture comprised 95 µL of ethanolic solution of DPPH and 5 µL of the test compounds, dissolved in DMSO. The total reaction volume was 100 µL, having final concentrations of 300 µM and 1000 µM of DPPH and test compounds, respectively. The reaction mixture was then incubated at 37° C for half an hour. After incubation, decrease in absorption was measured at 515 nm using multiplate reader (Spectra MAX-340). The control contained 5 µL of DMSO, instead of the test compound. The reactions were performed in triplicate. To avoid the evaporation of solvent during incubation, the 96-well plates were covered with parafilm immediately after the addition of DPPH solution and the reaction mixtures were thoroughly mixed by shaking the plate for one minute.

8.2.2. Superoxide Anion Scavenging Assay

Superoxide scavenging activities of compounds were determined by using the method described by N. S. C. Gaulejac (Gaulejac *et al.*, 1999) with some modifications.

8.2.2.1. Principle

The assay involves a non-enzymatic generation of superoxide anions ($O_2^{\bullet-}$). The superoxide anion scavenging activity was determined by measuring the reduction in rate of formation of formazan dye, which is blue in color and absorbs at 560 nm. It has been suggested that $O_2^{\bullet-}$ are generated when the 5-methyl phenazinium cation (PM^+) is reduced by NADH into 10-hydro-5-methylphenazine (PMH) and is then re-oxidized by the oxygen present in the atmosphere. The resultant $O_2^{\bullet-}$reduces the 2,2'-

di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethyl-4,4'-biphenylene)-ditetrazolium chloride (NBT or nitroblue tetrazolium salt) into the formazan dye. The trapping of $O_2^{\bullet-}$ by the test compounds inhibits the formation of formazan dye, which consequently decreases absorption at 560 nm (Soares *et al.*, 1997; Ishiyama *et al.*, 1993; Halaka *et al.*, 1982; Richter *et al.*, 1982;). The complete mechanism of action of the reaction is presented in Figure 8.2.

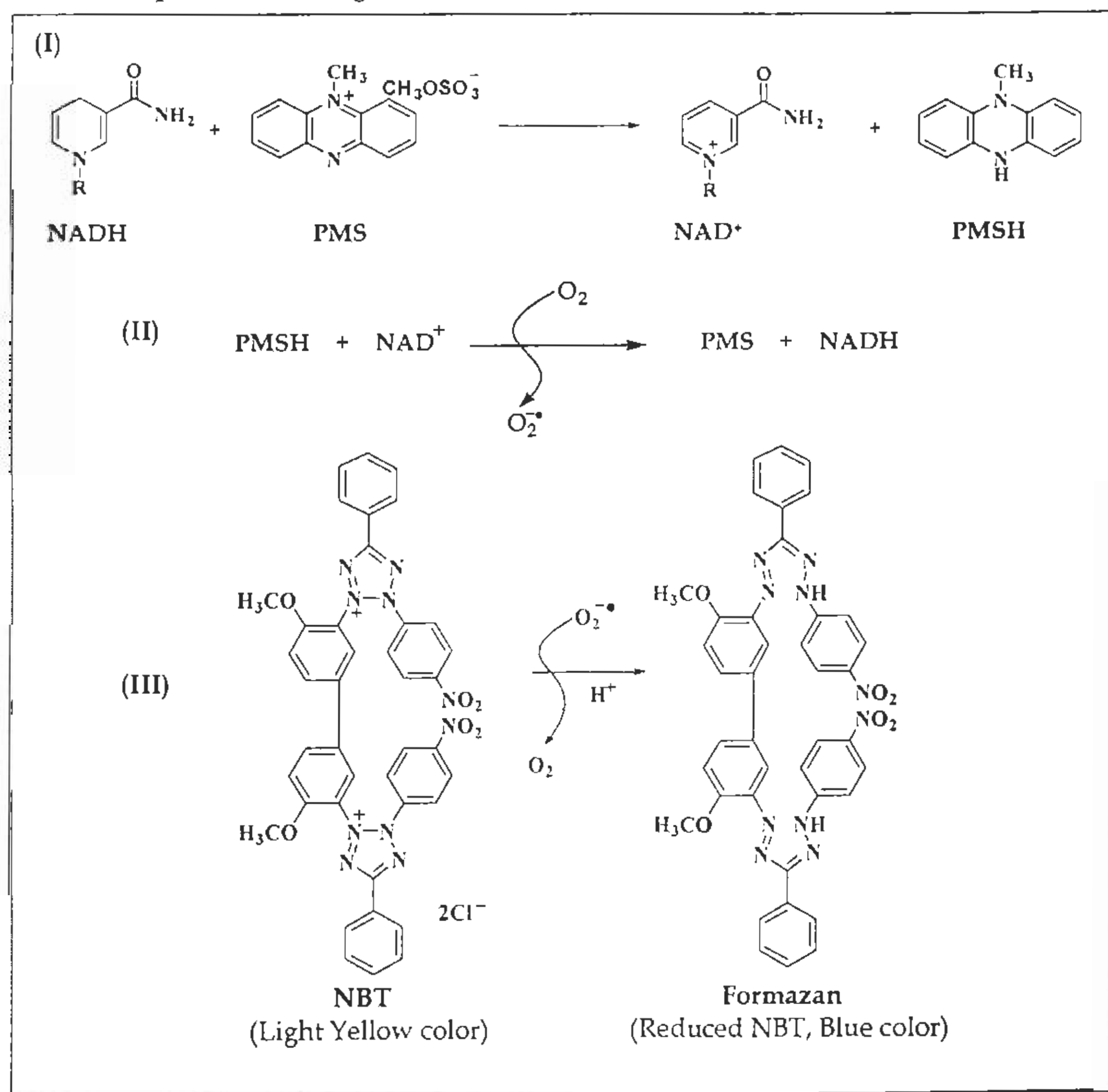

Figure 8.2: Mechanism of superoxide anions generation *via* NADH/PMS/O_2 system. (I) Reduction of PMS by NADH yields reduced phenazine (PMSH). (II) PMH again converted into PMS in the presence of oxygen and NAD^+, affording $O_2^{\bullet-}$. (III) Reduction of NBT with $O_2^{\bullet-}$produces a blue color formazan dye.

8.2.2.2. Method

The reaction was performed in triplicate in a 96-well plate and the absorbance was measured on multiplate reader (Sepctra MAX-340). The reaction mixture contained 40 μL nicotinamide adenine dinucleotide, reduced form (NADH), 40 μL nitroblue tetrazolium (NBT), 90 μL of 0.1 M phosphate buffer pH 7.5 and 10 μL of the test compounds. The reaction was initiated by the addition of 20 μL of phenazine methosulphate (PMS). The reaction temperature was maintained at 28° C. Formation of blue color formazan dye was measured after three minutes at 560 nm. The control contained 10 μL of DMSO instead of the test compound. The final concentrations of NADH, nitroblue tetrazolim salt (NBT) and PMS, in total reaction mixture volume of 200 μL, were 280, 80, and 8 μM, respectively, while the concentration of tested compounds was kept 1000 μM. The solutions of NBT, NADH and PMS were prepared in phosphate buffer while the test compounds were dissolved in DMSO.

8.2.2.3. Calculation of Radical Scavenging Activities

The DPPH radical scavenging and superoxide scavenging activities (%) were calculated by using the formula:

$$\% \mathbf{RSA} = [100-(\mathbf{AS}/\mathbf{AC}) \times 100]$$

where the **RSA** is radical scavenging activity, **AS** is the absorbance of DPPH radicals and formazan dye in the presence of test sample, and **AC** is the absorbance of DPPH radicals and formazan dye without sample (control).

8.2.2.4. IC_{50} Determinations

The IC_{50} (the concentration of the compound at which 50% radicals are scavenged or inhibited by the test compound) of all the compounds was determined by monitoring the effect of different concentrations ranging from 1-1000 μM. The IC_{50} of the compounds were calculated using EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc. Amherst, U. S. A.).

The xanthine oxidase inhibition potential of the test compounds was compared with allopurinol as standards.

8.2.3. Xanthine Oxidase (Butter milk) Inhibition Assay

Xanthine oxidase (EC 1.1.3.22) is a cytosolic molybdenum containing iron sulfur flavoprotein, which catalyzes the oxidative hydroxylation of a broad range of aldehydes and aromatic heterocyclics (Ryan *et al.*, 1995). Under certain pathophysiological conditions, xanthine oxidase (XO) can be a major source of $O_2^{\bullet -}$ intracellular production. It is a known source of free radicals in polymorphonuclear, endothelial, epithelial and connective tissue cells (Chung *et al.*, 1997).

8.2.3.1. Principle

Xanthine oxidase utilizes hypoxanthine or xanthine as a substrate and oxygen as a cofactor. The inhibitory activities of different classes of compounds against XO were measured by using a modified method of S. K. Lee (Lee *et al.*, 1998). The enzyme activity was determined by measuring the rate of hydroxylation of the substrate (xanthine) with the formation of uric acid, which is a colorless end product of the reaction and shows absorption at 295 nm.

The reaction catalyzed by XO is illustrated in Figure **8.3** (Richardson and Finley, 1997).

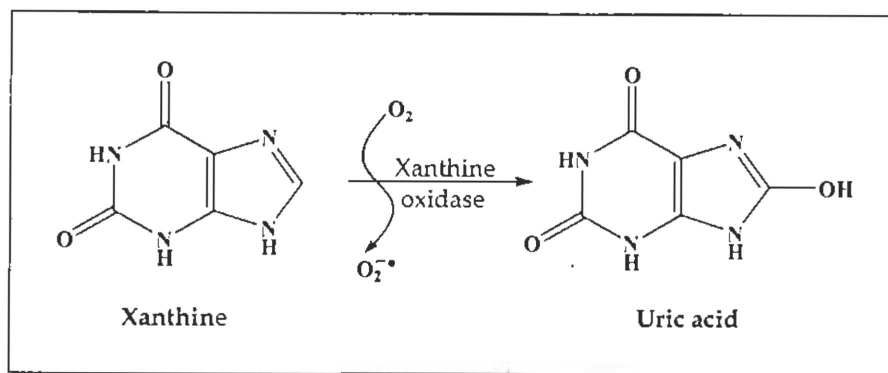

Figure 8.3. Mechanism of XO catalyzed oxidation of xanthine to uric acid.

8.2.3.2. Method

Xanthine solution was initially dissolved in a small volume of 0.1 N NaOH which was then made up with phosphate buffer upto the required volume. The inhibition of the enzyme xanthine oxidase (XO) by different compounds was determined by the method of S. K. Lee (Lee *et al.*, 1998) with the following modifications: the reaction mixture containing 20 μL of enzyme (0.003 U/200 μL), 10 μL of the test compound and 150 μL of 0.1 M phosphate buffer (pH 7.5) was incubated for ten minutes at 28° C. The reaction was initiated by the addition of 20 μL of substrate xanthine (100 μM), and the formation of uric acid was measured continuously at 295 nm for ten minutes on a multiplate reader (Spectra MAX-384). The final concentration of the test compound in the reaction mixture having total reaction volume 200 μL was 1000 μM. The reaction for each compound was again performed

in triplicate. The enzyme solution was prepared in phosphate buffer while the compound was dissolved in DMSO. To compare the inhibitory activities of the compounds, allopurinol was used as standard.

8.2.3.3. Calculations of Inhibitory Activities

The enzyme inhibitory activities were calculated using the following formula:

$$\% \text{ Inhibition} = [100-(S/E)] \times 100$$

where **S** is the activity of enzyme with the test sample and **E** is the activity of enzyme without sample.

8.2.3.4. IC_{50} Determinations

The IC_{50} of the compounds was determined by monitoring the inhibitory effect of different concentrations ranging from 100-1000 μM on the oxidation of xanthine. The IC_{50} of the compounds as calculated using EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc. Amherst, U. S. A.).

8.2.3.5. Mechanistic Studies

The enzyme (0.003 U/300 μL) was incubated with different concentrations of inhibitor for 10 min at 28° C. The reaction was then initiated by adding four different concentrations (7.14-20 μM) of substrate, xanthine and the resulting formation of uric acid was measured continuously at 295 nm for 80 sec. on a multiplate reader (Spectra MAX-384). One experiment was run in triplicate.

8.2.3.6. Determination of Type of Inhibition

The type of inhibition was determined by plotting the Lineweaver Burk, Dixon and their secondary plots. K_i Values (the dissociation constant of the enzyme inhibitor complex into free enzyme and inhibitor) were determined by the interpretation of Dixon plot (Dixon, 1953) and secondary plots of Lineweaver Burk (Segel, 1975) plots. The reciprocal of the rate of reactions against the reciprocal of the substrate concentrations and inhibitor concentrations were plotted as Lineweaver Burk and Dixon plots, respectively. In the case of un-competitive inhibition, the secondary plots of Lineweaver Burk plot were constructed by plotting the $1/V_{maxapp}$ and $1/K_{mapp}$ against the inhibitor concentrations. However, the values of K_{mapp} were determined from the line intersection on X-axis whereas the values of $1/V_{maxapp}$ were determined by the intersection point of every line on the Y-axis of Lineweaver Burk plot. For mixed-type inhibition, the secondary plots of Lineweaver Burk and Dixon were drawn by plotting the slope and $1/V_{maxapp}$ against the concentrations of respective inhibitor and slope against the concentrations of inhibitor, respectively. All the graphs were plotted using GraFit program (Leatherbarrow, R. J. GraFit; 4.09 ed.; Erithacus Software Ltd.; Tains, U. K.).

8.3. *In Vivo* Assay

The compounds, which showed lower IC_{50} against DPPH radicals and superoxide anions, were selected for *in vivo* study in the rat model of CCl_4-induced hepatotoxicity. The studies were carried out using the modified method of A. Kapil (Kapil and Koul, 1995).

Adult male Wistar rats (180-220 gm) were bred in the animal house of the H. E. J. Research Institute of Chemistry, University of Karachi, or purchased from the Aga Khan Medical University, Karachi. Animals were kept in 12 h light-12 h dark cycle and fed on a standard laboratory diet and tap water *ad libitum*.

8.3.1. Principle

It has been accepted that CCl_4 is converted into the trichloromethyl free radicals ($^{\bullet}CCl_3$) by the NADPH-cytochrome P-450 system, i.e. by transfer of an electron from NADPH to CCl_4 (Zhu and Fung, 2000). Studies suggested that resulting radical species ($^{\bullet}CCl_3$) react with the oxygen to form (trichloromethyl peroxyl radical) $CCl_3OO^{\bullet}$ (Connort *et al.*, 1986). $CCl_3OO^{\bullet}$ is reactive free radical capable of initiating lipid peroxidation of cellular and organelle membranes, which damage the integrity of bio-membranes. This leads to the release of large quantities of cytosolic enzymes into blood, causing swelling and necrosis of liver cells (Takahashi *et al.*, 1996). The estimation of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubins is largely used for the assessment of liver damage by free radicals generated by CCl_4. AST is present in liver, heart, muscle, kidney and the brain. It catalyzes the conversion of aspartate into oxaloacetate and glutamate. ALT catalyzes the conversion of alanine into pyruvate and glutamate. Necrosis or

membrane damage results in the release of these enzymes into blood circulation, which can be easily estimated (Ferreira *et al.*, 2003).

8.3.2. Method

Rats were divided into three groups of six animals. Group 1 was the normal control group, group 2 was pathological control group, and group 3 was the test group. Group 1 served as vehicle control and received normal saline only. Group 2 received i.p. dose of 20% CCl_4 diluted with dietary cooking oil (1 mL/100 g body weight). The test group 3 received test compounds, 10 mg/Kg body weight, p.o. After 30 minutes of administration of the compound, group 3 animals received the i.p. dose of 20% CCl_4 diluted with dietary cooking oil (1 mL/100 g body weight). All animals were sacrificed 48 hrs after CCl_4 administration and blood was collected in test tubes. The blood samples were allowed to clot for 45 minutes at room temperature. The serum was then separated by centrifugation at 3000 rpm at room temperature for 15 minutes and analyzed for various biochemical parameters. All the test compounds were dissolved either in water or in a mixture of DMSO/ water (5:95) depending on their solubility profile.

8.3.2.1. Determination of Serum Biochemical Parameters

The enzymes, AST, ALT, and total and direct bilirubin were analyzed by biochemical analysis of the serum using standard Kit method (Boehringer Mannheim).

8.3.2.2. Serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were analyzed by the kit method developed by Bergmeyer and Horder

(Bergmyer and Horder, 1986: Bergmeyer and Horder, 1980). A 2 mL of reagent solution, containing *tris* buffer (80 mM, pH 7.8); *L*-aspartate (240 mM); MDH (0.42 U/mL); LDH (0.60 U/mL); NADH (0.18 mM) and α-oxalogultarate (12 mM), was mixed with 2 mL of the serum and the changes in absorbance at 340 nm were recorded after 1 minute interval for 3 minutes. The value of AST in IU/L was determined by multiplying the mean absorbance by the factor 1746. The same procedure was used to measure the ALT by using the reagent solution having *tris* buffer (110 mM, pH 7.3); *L*-alanine (550 mM); LDH (1.3 U/mL); NADH (0.198 mM); α-oxalogultarate (15 mM).

8.3.2.3. Total Bilirubin concentration was measured by the method of Jendrassik and Grof and Sherlock (Sherlock, 1951; Jendrassik and Grof, 1938) using Randox Kit. The serum sample was mixed with reagent 1 (sulphanilic acid, 29 mM and HCl, 0.17 N), reagent 2 (sodium nitrite, 25 mM), reagent 3 (caffeine, 0.26 M and sodium benzoate, 0.52 M) and allowed to stand for 10 minutes. After ten minutes, reagent 4 (tartarate, 0.93 M and sodium hydroxide, 1.9 N) was added and the serum was incubated at 25° C for 30 minutes. Absorbance was then recorded at 578 nm against sample blank. The reaction was repeated three times and the resulting mean absorbance was multiplied with a factor 10.8 in order to determine the total bilirubin concentration (mg/dL) in serum.

8.3.2.4. Direct Bilirubin concentration was measured by using the same method as described above. Here reagent 1 (sulphanilic acid, 29 mM and HCl, 0.17 N) and one drop of reagent 2 (Sodium nitrite, 25 mM) were added to the serum. Then 2.0 mL of normal saline (0.9 %) was added instead of reagent 3. The serum was then allowed to

stand for 5 minutes and absorbance was measured at 546 against blank sample. The concentration of direct bilirubin in mg/dL was obtained by multiplying the mean absorbance of three experiments with the factor 14.4.

8.3.2.5. Indirect Bilirubin concentration was calculated by subtracting the direct bilirubin concentration from total bilirubin concentration as described below.

I. Bilirubin = T. Bilirubin – D. Bilirubin

where **I. Bilirubin** is the indirect or conjugated bilirubin, **T. Bilirubin** is total or conjugated and un-conjugated bilirubin and **D. Bilirubin** is direct or un-conjugated bilirubin.

8.3.2.6. Statistics

All data are expressed as mean ± standard deviation of the mean (SD). The results were evaluated by performing analysis of variance (ANOVA). Statistically significant differences were defined as values less than $P \leq 0.05$.

8.4. Cytotoxicity Assay

The cytotoxicity of compounds was evaluated by the method of Tan and Berridge (Tan and Berridge, 2000) with modifications. The freshly isolated human neutrophils were used instead of cell lines and the test compound was incubated with neutrophils in the presence of WST-1 tetrazolium salt (200 μM) for 3 hrs.

8.4.1. Principle

Neutrophils are the most abundant defensive cells against bacterial infections. Like all the cells of the immune system, these are produced in the bone marrow and circulated in the bloodstream. However, neutrophils move out of blood vessels into infected tissue in order to attack the foreign substance. They spend only a few hours in the bloodstream before being recruited toward inflammatory districts, where they survive for 1–2 days before undergoing spontaneous apoptosis. In the tissue, they act as phagocytic cells and also release a variety of reactive oxygen species (ROS) and proteases (Dona *et al.*, 2003).

The tetrazolium salt WST-1 (4-[3-(4-iodopheyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is reduced to yellow color water soluble formazan dye by the action of cellular dehydrogenases of viable cells. However, the metabolically inactive cells are unable to reduce WST-1. It is therefore in the presence of a toxic compound that the metabolic activities of cells are destroyed and no reduced WST-1 is produced (Hong *et al.*, 2001; Berridge *et al.*, 1996). The structure of reduced WST-1 is presented in Figure **8.4**.

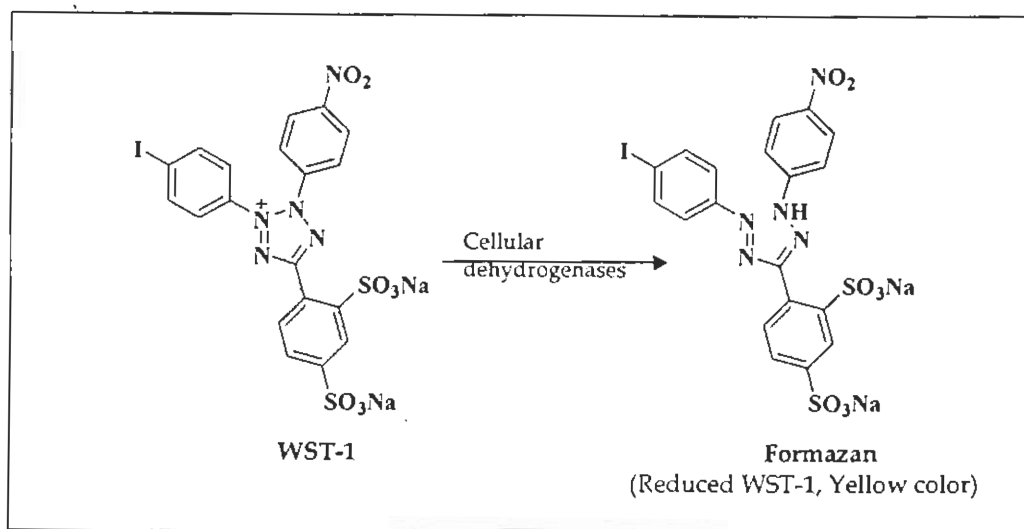

Figure 8.4 : Reduction of WST-1 tetrazolium salt by the action of cellular dehydrogenases.

8.4.2. Isolation of Neutrophils

Heparinized fresh venous blood was drawn from healthy volunteers in a blood bank (Fatmeed Foundation) and neutrophils were isolated by the method of Siddiqui *et al.* (Siddiqui *et al.*, 1995). Blood was mixed with ficoll paque in the ratio of 1:3 and allowed to settle down for 40-60 minutes at room temperature. Then the buffy coat was layered on the bed of the ficoll centrifuged at 1500 rpm for 30 min. The pallets, settled down in the test tube, were then collected and washed with PBS (pH 7.4). The RBCs were then lysed with ammonium chloride solution. Finally, the pallets were centrifuged for 30 min and then washed with same PBS (pH 7.4). The final concentration of cells up to 1x 10^6 cells/mL was maintained in PBS (pH 7.4). The cell count was carried out by using the Neubauer chamber.

8.4.3. Method

The cytotoxicity evaluation of test compounds was carried out in four replicates using 96-well plate and the absorbance of reduced WST-1 salt was measured on a multiplate reader (Spectra MAX-384). The various concentrations (12.5-200 μg/mL, 25 μL) of test compounds were incubated with neutrophils (1x 10^6 cells/mL, 150 μL) at 37° C for 30 min. The WST-1 tetrazolium salt (200 μM, 25 μL) was then added to the reaction mixture and background absorbance was measured at 450 nm. The reaction mixture was then incubated for 3 hrs at 37° C in a shaking water bath. After incubation, the final absorbance was measured at 450 nm. The sample solutions were initially dissolved in DMSO and the required concentration was maintained using MHS (pH 7.4). The ratio of DMSO and MHS was 5: 95. The positive control contained buffer, cells and WST-1 salt, whereas the negative control had 0.1 % triton X-100.

8.4.4. Determination of Cell Viability %

The cell viability (%) was calculated using following formula:

$$\text{\% Cell viability} = \frac{AS}{AC} \times 100$$

where **AS** (absorbance of sample) is the absorbance of yellow color formazan in the presence of test compound, while **AC** (absorbance of control) is the absorbance in the absence of test compound.