SECTION III

EXPERIMENTAL WORK
CHAPTER 6

Materials And Methods
EXPERIMENTAL WORK

Introduction

Oxidative stress is thought to be a key event in the pathogenesis of reperfusion injury. Overproduction of reactive oxygen species during I/R could cause an imbalance between oxidative and antioxidative processes. Reactive oxygen species can damage lipids, proteins, and nucleic acids, thereby inducing apoptosis or necrosis. Thus antioxidants can protect against the adverse effects of free radical production during ischemia and reperfusion [1-6].

Pyrimidines exert a variety of biochemical and pharmacological effects. Recently, considerable interest has been focused on pyrimidines because of their antioxidant, anti-inflammatory, and antiproliferative activities. There is an increasing evidence supporting the hypothesis that pyrimidines can provide protection against neurodegenerative changes associated with cerebral ischemia and reperfusion injury. Pyrimidines are reported to have beneficial effects on health due to their antioxidant function. This effect of pyrimidines therapy on cerebral reperfusion injury has not yet been completely elucidated. Therefore in the present study cerebroprotective actions of selected pyrimidine derivatives were evaluated against I/R induced cerebral infarction in rats [7-10].
MATERIALS AND METHODS

Pyrimidines

The pyrimidines (Pyrimidine 1 and Pyrimidine 2) considered in the present study were synthesized and characterized in Pharmaceutical Chemistry Research Laboratories, AU College of Pharmaceutical Sciences, Andhra University. The physical and spectral characterization data of pyrimidines (1 and 2) have given one by one. The 2D molecular representation of pyrimidines is shown below.
Pyrimidine 1

Nomenclature : 2-amino-4-(4'-aminophenyl)-6-(3''-bromophenyl) pyrimidine

Molecular formula : C_{16}H_{13}N_{4}Br

Molecular weight : 341

Melting point : 230±2°C

Yield (%) : 45.28

R_{f} : 0.56 (40% ethylacetate/hexane)

IR (KBr, cm\(^{-1}\)) : 3401, 3305 (NH\(_2\)), 1635 (C=N), 1575 (C=C), 1362 (C=N), 578 (C-Br)

\(^1\)H-NMR (δ ppm) : 5.65 (2H, s, C-4'-NH\(_2\)), 6.55 (2H, s, C-2-NH\(_2\)), 6.65 (2H, d, J=8.4 Hz, C-3' and 5'-H), 7.49-7.45 (1H, t, C-5''-H), 7.58 (1H, s, C-5-H), 7.69 (1H, d, J=8.8 Hz, C-6''-H), 7.99 (2H, d, J=8.4 Hz, C-2' and 6'-H), 8.20 (1H, d, J=8.0 Hz, C-4''-H), 8.41 (1H, s, C-2''-H)

Elemental analysis (%) :

\[
\begin{array}{ccc}
\text{C} & \text{H} & \text{N} \\
\text{Calculated} & 56.35 & 3.84 & 16.43 \\
\text{Found} & 56.30 & 3.81 & 16.42 \\
\end{array}
\]
Pyrimidine 2

Nomenclature : 2-amino-4-(4'-aminophenyl)-6-(4''-methoxyphenyl) pyrimidine

Molecular formula : C_{17}H_{16}N_{4}O

Molecular weight : 292

Melting point : 248±2°C

Yield (%) : 37.01

R_f : 0.55 (40% ethylacetate/hexane)

IR (KBr, cm⁻¹) : 3440, 3309 (NH₂), 1614 (C=N), 1575 (C=C), 1364 (C-N), 1238 (C-O-C)

¹H-NMR (δ ppm) : 3.84 (3H, s, C-4''-OCH₃), 5.60 (2H, s, C-4'-NH₂), 6.39 (2H, s, C-2-NH₂), 6.64 (2H, d, J=8.4 Hz, C-3' and 5'-H), 7.04 (2H, d, J=8.8 Hz, C-3'' and 5''-H), 7.46 (1H, s, C-5-H), 7.95 (2H, d, J=8.4 Hz, C-2' and 6'-H), 8.15 (2H, d, J=8.8 Hz, C-2'' and 6''-H)

Elemental analysis (%) : 

<table>
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<td>19.18</td>
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<tr>
<td>Found</td>
<td>69.86</td>
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Animals

All experimental protocols were approved by the Institutional Animal Ethics Committee of AU College of Pharmaceutical Sciences, Andhra University vide proposal no: (Approval No. 516/01/A/CPCSEA) under the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. Wistar rats weighing 250-300 g of either sex were used which were obtained from National Institute of Nutrition, Hyderabad. Animals were housed in groups of 6-7 in colony cages at an ambient temperature of 25±2 °C and 45-55% relative humidity with 12 h light/dark cycle. They had free access to pellet chow (Pranav Agro Limited) and water *ad libitum*.
### Chemicals

<table>
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<tr>
<th>SI.No</th>
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<tbody>
<tr>
<td>1.</td>
<td>Pyrimidine 1</td>
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</tr>
<tr>
<td>2.</td>
<td>Pyrimidine 2</td>
<td>Synthesized</td>
</tr>
<tr>
<td>3.</td>
<td>Triphenyl tetrazolium chloride (TTC)</td>
<td>Himedia</td>
</tr>
<tr>
<td>4.</td>
<td>1,1,3→3-tetraethoxy propane (TEP)</td>
<td>Sigma</td>
</tr>
<tr>
<td>5.</td>
<td>Thiobarbituric acid</td>
<td>Loba Chemicals</td>
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<tr>
<td>6.</td>
<td>Sodium dodecyl sulphate</td>
<td>SD Fine Chemicals</td>
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<tr>
<td>7.</td>
<td>n-Butanol</td>
<td>SD Fine Chemicals</td>
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<td>9.</td>
<td>Phenazine methosulphate</td>
<td>Loba Chemicals</td>
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<tr>
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<td>Nitroblue tetrazolium</td>
<td>SD Fine Chemicals</td>
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<td>NADH</td>
<td>Sisco Chemicals</td>
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<td>Hydrogen peroxide</td>
<td>Merck</td>
</tr>
<tr>
<td>13.</td>
<td>L-NAME</td>
<td>Sigma</td>
</tr>
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<td>14.</td>
<td>Hexadecyl trimethyl ammonium bromide</td>
<td>SD Fine Chemicals</td>
</tr>
<tr>
<td>15.</td>
<td>O-dianisidine dihydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>16.</td>
<td>Allopurinol</td>
<td>Strides Arcolab</td>
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</table>

And all other chemicals and solvents used were of analytical grade purchased from commercial suppliers
**Induction Of Cerebral Infarction**

Overnight fasted rats were anaesthetized with thiopental sodium (30 mg/kg). A midline ventral incision was made in the throat. Right and left common carotid arteries were located and freed from surrounding tissue and vagus nerve. A cotton thread was passed below each of the carotid artery. Global cerebral ischemia was induced by occluding the carotid arteries by a knot. After 30 min of global cerebral ischemia, the cotton threads were removed with the help of two knot releasers to allow the reperfusion of blood through carotid arteries for 4 h. Body temperature of rats was maintained at 37 °C by heated surgical platform. All surgical procedures were carried out under sterile conditions. [11]

**Determination Of Infarct Size**

The infarct size was determined in rats from the experiments as described previously. In brief, animals were killed at the end 4 h reperfusion and brains were removed rapidly by cervical dislocation and frozen at -4 °C for 5 min. Coronal slices were made at 1-2 mm and sections were immersed in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37 °C for 20 min. TTC is converted to red formazone pigment by NAD and dehydrogenase present in living cells. Hence viable cells were stained deep red. The infarcted cells have lost the enzymes and thus remained unstained. Whole brain slices were weighed. Infarcted unstained part was dissected out, weighed and expressed as % of total weight of brain.
Estimation Of Biochemical Parameters

Preparation of brain tissue for estimation of oxidative stress markers: Stained tissue were not suitable for estimating the oxidative stress markers, hence a separate group of animals were used for estimating the these enzymes. The brain of each animal was removed after completion of 4 h reperfusion following decapitation and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phosphate buffer (0.1M, pH 7.4) using a Remi homogenizer. Homogenisation procedure was performed as quickly as possible under completely standardized conditions. The homogenate was centrifuged at 1000 rpm 4 °C for 3 min and the supernatant divided into two portions, one of which was used for measurement of malondialdehyde (MDA). The remaining supernatant was again centrifuged at 12,000 rpm at 4 °C for 15 min and used for the measurement of superoxide dismutase (SOD), catalase (CAT). Protein was measured by the method of Lowry et al (1951) [12].

Estimation Of Malondialdehyde

Lipid peroxidation is a well-defined mechanism of cellular damage in animals and plants. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural byproducts of lipid peroxidation. Oxidative modification of lipids can be induced in vitro by a wide array of pro-oxidant agents and occurs in vivo during aging and in certain disease conditions. Measuring the end products of lipid peroxidation is one of the
most widely accepted assays for oxidative damage. These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress.

Thiobarbituric Acid Reactive Substances (TBARS) is a well-established assay for screening and monitoring lipid peroxidation.

The assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at 95 °C in presence of acetic acid; forming a MDA-TBA$_2$ adduct that absorbs strongly at 532 nm.

![Chemical structure of TBARS reaction](image)

**Reagents**

1) **Potassium chloride (1.15%) solution:**

1.15 g of Potassium chloride was weighed, transferred to a volumetric flask and the volume was made up to 100 mL with distilled water.

2) **Thiobarbituric acid (0.8%) solution:**

0.8 g of Thiobarbituric acid was weighed transferred to a volumetric flask and the volume was made up to 100 mL with distilled water.

3) **Sodium dodecyl sulphate (8.1%) solution:**

8.1 g of Sodium dodecyl sulphate was weighed transferred to a volumetric flask and the volume was made to 100 mL with distilled water.
4) **Acetic acid (20%) solution:**

20 mL of Glacial acetic acid was transferred to a volumetric flask and the volume was made to 100 mL with distilled water. The pH was adjusted to 3.5 with drop wise addition of N/10 Sodium hydroxide if necessary.

**Preparation Of Standard Curve**

**Procedure [13]**

Stock solution of 1, 1, 3, 3-tetraethoxypropane (TEP) was prepared by taking 22 mg of TEP in a volumetric flask and the volume was made up to 10 mL with distilled water in a volumetric flask.

- From this stock solution, 1 mL was taken in a volumetric flask and volume was made up to 100 mL with distilled water to obtain a 10 μM solution.
- From this working standard solution 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL quantities representing 2 nmol, 4 nmol, 6 nmol, 8 nmol and 10 nmol of TEP, respectively were transferred into centrifuge tubes.
- Then 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid solution, and 1.5 mL of 0.8 % aqueous solution of TBA were added and mixed thoroughly.
- The final volume in all the tubes was made up to 5 mL with distilled water. Then the reactants were heated in oil bath at 95 °C for about 60 min.
- After the tubes were cooled to room temperature and the resulting chromogen was extracted with 5 mL of 15:1 v/v n-butanol and pyridine
mixture by vigorous shaking.

- Separation of the organic phases was facilitated by centrifugation at 4000 rpm for 10 min and its absorbance was measured at 532 nm.
- The standard graph was prepared by taking concentration of MDA on x-axis and the corresponding absorbance on y-axis.
- All the points are in same line showing a linear relationship between the absorbance of colored solution and MDA concentration ranging from 2 nmol to 8 nmol.
- The standard curve in the above range if following Beer-Lambert's law. The calibration curve is shown in Table 6.1 and Fig. 6.1.

**Table 6.1.** Optical density values for plotting calibration curves for the estimation of malondialdehyde in brain tissue.

<table>
<thead>
<tr>
<th>SI.No</th>
<th>Tetraethoxypropane (n.mol)</th>
<th>OPTICAL DENSITY</th>
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<td>Trial-1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>0.159</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
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<tr>
<td>4</td>
<td>0.6</td>
<td>0.407</td>
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<tr>
<td>5</td>
<td>0.8</td>
<td>0.533</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.681</td>
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</table>
Procedure For Sample

- MDA levels in the brain homogenate were measured by the method developed by Ohkawa et al.
- To a sample of 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid solution, and 1.5 mL of 0.8% aqueous solution of TBA were added.
- The mixture was made up to 5 mL with distilled water and then heated in an oil bath at 95 °C for 60 min using a glass ball as a condenser.
- After cooling with tap water, 5 mL of mixture of n-butanol and pyridine (15:1 v/v) was added and shaken vigorously.
- After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance was measured at 532 nm.
- The tissue MDA levels were measured from the standard curve and expressed as nmol/g tissue.
Estimation Of Superoxide Dismutase (SOD)

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, I/R injury, atherosclerosis and aging. Superoxide Dismutases (SODs) catalyze the dismutation of the superoxide radical \( O_2^- \) into hydrogen peroxide \( H_2O_2 \) and elemental oxygen \( O_2 \) which diffuses into the intermembrane space or mitochondrial matrix and thus, SODs provide an important defense against the toxicity of superoxide radicals.

In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use and it is used in the present study.

Reagents

1) **Sodium pyrophosphate buffer (0.052 M, pH 8.3) Solution:**

   1.38 g of Tetra-sodium pyrophosphate was weighed transferred to a volumetric flask and the volume made up to 100 mL with distilled water and the pH was adjusted to 8.3.

2) **Phenazine methosulphate (186 μM) solution:**

   5.69 mg of Phenazine methosulphate was weighed and transferred to a volumetric flask and the volume made up to 100 mL with distilled water.

3) **Nitroblue tetrazolium (300 μM) solution:**

   12.25 mg of Nitroblue tetrazolium was weighed and transferred to a volumetric flask and the volume made up to 50 mL with distilled water.
4) **NADH (780 μM) solution:**

13.83 mg of NADH was weighed, transferred to a volumetric flask and the volume made up to 25 mL with distilled water.

**Procedure [14]**

- Superoxide dismutase (SOD) activity was determined by the method developed by Kakkar et al.
- Brain tissue was homogenized with Remi homogenizer in ice cold phosphate buffer (0.1M, pH 7.4) to produce a 10% w/v homogenate.
- The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C
- Aliquot of 0.1 mL supernatant was added to 1.2 mL of 0.052 M sodium pyrophosphate buffer (pH 8.3) followed by addition of 0.1 mL of 186 μM phenazine methosulphate, 0.3 mL of 300 μM nitroblue tetrazolium, 0.2 mL of 780 μM NADH.
- Reaction mixture was incubated for 90 sec at 30 °C, and the reaction was stopped by the addition of 0.1 mL of glacial acetic acid.
- Reaction mixture was stirred vigorously and shaken with 4.0 mL of n-butanol and centrifuged at 4000 rpm for 10 min.
- The absorbance of organic layer was measured at 560 nm. A control was prepared using 0.1 mL of distilled water devoid of 0.1 mL of homogenate.
- One unit of the enzyme activity is defined, as enzyme concentration required inhibiting the absorbance of chromogen production by 50% in control sample under the assay conditions. The SOD level was expressed as Units/ mg protein.
Estimation Of Catalase (CAT)

Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system. It was initially isolated from ox liver and later from blood, bacterial, and plant sources. The enzyme contains 4 ferrihemoprotein groups per molecule. The enzyme has a molecular mass of 240 kDa. Catalase activity varies greatly between tissues. Catalase catalyses the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. Hydrogen peroxide is formed in the eukaryotic cell as a byproduct of various oxidase and superoxide dismutase reactions. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death. Removal of the H$_2$O$_2$ from the cell by catalase provides protection against oxidative damage to the cell. It's role in oxidative stress related diseases has been widely studied.

Reagents

1) **Potassium phosphate buffer (50mM, pH 7.0) solution:**

   871 mg of Dipotassium hydrogen phosphate and 680 mg of potassium dihydrogen phosphate were weighed, transferred to a volumetric flask and the volume made up to 100 mL with distilled water and the pH was adjusted to 7.0.

2) **Hydrogen peroxide (30 mM) solution:**

   34 μL of 30% Hydrogen peroxide was transferred to a volumetric flask and the volume made up to 10 mL with distilled water.
**Procedure** [15]

- Catalase activity was measured by the method of Aebi. Brain tissue was homogenized with a Remi homogenizer in ice-cold phosphate buffer (0.1M, pH 7.4) to produce a 10% w/v homogenate.
- The homogenate was centrifuged at 12,000 rpm at 4°C for 15 min. Supernatant 0.1 mL was added to cuvette containing 1.9 mL of 50 Mm phosphate buffer.
- To this mixture, 1.0 mL of freshly prepared 30 mM H$_2$O$_2$ was added and changes in absorbance for 3 min at 240 nm at an interval of 30 sec.
- A control was prepared using 0.1 mL of distilled water devoid of 0.1 mL of homogenate. Activity of catalase was expressed as μmoles of H$_2$O$_2$ metabolized/mg protein/min.

**Estimation Of Myeloperoxidase (MPO)**

Myeloperoxidase (MPO) is a highly cationic glycosolated hemoprotein that has a molecular weight of 144kD. The hemoprotein consists of two dimers linked via a disulfide bridge. Each dimer is composed of a heavy (53kD) and light (15kD) subunit. Each heavy chain contains an independently acting protoporphyrin group containing a central iron. MPO is present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one third of the MPO found in PMN's. MPO utilizes H$_2$O$_2$ produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals. This enzyme is unique however
in that it can oxidize chloride ions to produce a strong non-radical oxidant, HOC1. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury.

Reagents

1) **Phosphate buffer (50 mM, pH 6.0) solution:**

   **Solution A:** 138 mg of NaH$_2$PO$_4$ was weighed, transferred to a volumetric flask and the volume made up to 100 mL with distilled water.

   **Solution B:** 284 mg of Na$_2$HPO$_4$ was weighed, transferred to a volumetric flask and the volume made up to 100 mL with distilled water. From the above solutions, 12 mL of solution A and 88 mL of solution B were mixed and pH was adjusted to 6.0.

2) **Phosphate buffer containing 0.5% hexadecyl trimethylammonium bromide solution:**

   500 mg of hexadecyl trimethylammonium bromide was weighed and transferred into a volumetric flask and the volume made up to 100 mL with distilled water.

3) **Hydrogen peroxide (0.05%) solution:**

   167 μl of 30% of hydrogen peroxide was transferred into a volumetric flask and the volume made up to 100 mL with distilled water to obtain a 0.05% solution.

4) **Phosphate buffer containing O-dianisidine dihydrochloride (0.167 mg/mL) and hydrogen peroxide (0.0005%) solution:**
16.7 mg of o-dianisidine dihydrochloride and 1 mL of 0.05% of hydrogen peroxide were transferred into a volumetric flask and the volume made up to 100 mL with distilled water.

**Procedure [16]**

- The activity of myeloperoxidase was assessed using the method modified from that of Mullane et al.
- The brain tissue was homogenized in phosphate buffer containing 0.5% hexadecyl trimethylammonium bromide using a Polytron homogenizer to produce a 10% w/v homogenate.
- After freeze-thawing for three times, the samples were centrifuged at 15000 rpm for 30 min at 4°C and the resulting supernatant assayed spectrophotometrically for MPO.
- To 40 μL of the sample, 960 μL of phosphate buffer containing o-dianisidine dihydrochloride and hydrogen peroxide was mixed and shaken vigorously.
- The change in the absorbance was measured at 460 nm for 3 min at an interval of 60 sec.
- One unit of enzyme activity was defined as the amount of MPO that causes a change in absorbance measured at 460 nm for 3 min. Myeloperoxidase activity was expressed as U/g tissue.

**Estimation Of Tumor Necrosis Factor-Alpha (TNF-α)**

Tumor necrosis factor-alpha (TNF-α) is a potent cytokine with a myriad of innate immune anti-tumor properties. TNF-α has a critical role in the bone and
cartilage damage associated with rheumatoid arthritis (RA). TNF-α may be involved in the pathogenesis and/or progression of gestational diabetes mellitus (GDM). TNF-α is expressed in myocardium during compensated pressure-overload hypertrophy and contributes to post-ischemic myocardial dysfunction. The serum levels of TNF-α were also significantly elevated in active WG (Wegener's granulomatosis), in the late stages of HIV-associated disease, and in the spinal cord of arthritic patients.

**Reagents And Procedure** [17]

- AssayMax Rat Tumor Necrosis Factor alpha (TNF-alpha) ELISA Kit.
- Catalog No. ERT2010-1.

**Estimation Of Interleukin-10 (IL-10)**

Interleukin-10 (IL-10) is a regulatory cytokine, and its principal role *in vivo* is to limit inflammatory response. IL-10 has been shown to influence both the susceptibility and course of various diseases. High IL-10 expression whereas monocytes from cardiac-disease, patients may be committed to induction of inflammatory responses related to high TNF-α expression. Interleukin 10 (IL-10) is a key cytokine produced by a multitude of immune effector cells and possesses distinct regulatory effects on immune functioning in the skin. The accelerated alveolar bone loss observed in IL-10 (-/-) mice is a late-onset condition and that lack of IL-10 may have an effect on bone homeostasis.

**Reagents And Procedure** [18]

- AssayMax Rat Interleukin-10 (IL-10) ELISA Kit.
- Catalog No. ERI3010-1.
**Statistical Analysis**

The results were expressed as (Mean± SEM). Differences in infarct size, MDA, SOD, CAT, MPO, TNF-α and IL-10 were determined by factorial One-way ANOVA. Individual groups were compared using Tukey’s test. Differences with P<0.05 were considered statistically significant. Statistical analysis was performed using Prism software (Version 6.02).
REFERENCES


[18] AssayMax Rat Interleukin-10 (IL-10) ELISA Kit: Catalog No. ERI3010-1.