CHAPTER 2

EVALUATION OF
BIOCHEMICAL PARAMETERS
OF MACROPHAGE

A] ASSESSMENT OF REACTIVE NITROGEN AND
REACTIVE OXYGEN INTERMEDIATES

B] ASSESSMENT OF ENZYMES
CHAPTER 2 EVALUATION OF BIOCHEMICAL PARAMETERS OF MACROPHAGE ACTIVATION

Abstract:

The chapter deals with assessment of reactive oxygen and nitrogen intermediates produced by macrophages treated with medium alone or BRMs or LPS. Increased secretion of reactive nitrogen intermediates i.e. nitrite (NO); reactive oxygen intermediates i.e. hydrogen peroxide (H₂O₂) and superoxide (O²⁻) was observed. Also enhanced production of enzymes like NADH-NADPH oxidase, myeloperoxidase (MPO) and lysozyme by macrophages treated with BRMs and LPS was observed. It is suggested that increased production of these secretory products represent the activated state of macrophages. These in vitro activated macrophages could either enhance phagocytosis or kill the tumor cells via cytolysis mediated by the released secretory products.

2.1 Introduction:

Macrophages play a crucial role in host defense by generating microbicidal oxygen metabolites including superoxide anions (O²⁻), hydrogen peroxide (H₂O₂) and hydroxyl ions (Babior, 1978). Macrophage activation is known to occur through a series of stages, which results in the production of biologically active molecules such as the reactive oxygen and nitrogen intermediates. The generation of reactive oxygen and nitrogen intermediates can be measured spectrophotometrically.

The study is divided into two parts i.e.: A] Assessment of Reactive nitrogen and oxygen intermediates B] Assessment of enzymes involved in respiratory burst.

Macrophages (J774A.1) treated with BRMs and LPS showed an enhanced production of nitrite, superoxide and hydrogen peroxide. Also enhanced enzyme production in respiratory burst was observed i.e. lysozyme NADH-NADPH and myeloperoxidase.
2.1 (a) Generation of nitrite: NO has been the center of attraction for the scientists since the 18th century. NO has a dual role of acting as 2nd messenger and maintaining the physiologic condition in healthy people and even playing an important role during inflammation. NO is synthesized through the activity of nitric oxide synthase (NOS) enzyme. NOS enzyme exists in three different isoforms i.e. NOS1 or neuronal NOS (nNOS), NOS2 or inducible NOS (iNOS) and NOS3 or endothelial NOS (eNOS) all of which are 125 – 160 kD heterodimers with >50% sequence identity (Marletta, 1993). These NOS isoforms are differentially expressed and regulated, and have several important physiological and pathophysiological roles ranging regulation of vascular tone (Massion, 2003; Münzel, 2005) to inflammation and cancer (Ying, 2007; Wink et al., 1998). Nitric oxide is highly reactive, with multiple products, including peroxynitrate, nitrate and nitrite (Fig15). Quantification of systemic NO production typically involves conversion of nitrate to nitrite followed by spectrophotometric quantification using the Griess reaction.
To investigate the key role of NOS in many tissues, several studies involve analysis of NOS activity in tissue culture. Given the relatively small quantities of NOS in these systems, analysis of NOS activity has typically been performed by measurement of the conversion of radiolabeled L-arginine to L-citrulline. This radioisotopic method is quantitative, but the use of radiolabeled materials and the need to isolate and quantify the L-citrulline product makes them relatively expensive and time-consuming. By using Griess reagent a validated and a non-radioactive yet ultrasensitive colorimetric nitrite assay was standardized (Ghigo et al., 2006). In this method NADPH employs NOS to catalyze NO production. The stable NO degradation products, nitrate and nitrite accumulate during this period. Following enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase (NaR), the nitrite concentration is determined using the Griess Reagent. This assay is very suitable for the efficient high-throughput screening of nitrite in biological fluids.

2.1 (b) Superoxide: With the invasion of host cells by any pathogen a series of host defensive mechanisms come into play. The 1st preventive step taken by the cells is inflammation leading to the activation of macrophages. The initial step of macrophage activation involves phagocytosis leading to the respiratory burst which in turn
activates membrane bound oxidases like NADPH oxidase. NADPH oxidase further catalyses the reduction of \( O_2 \) to superoxide anion, a reactive oxygen intermediate toxic to ingested microbes by causing severe oxidative stress (Forman and Martine, 2001). Hence, superoxide has been shown to have several effects as in low or sub lethal doses it is involved in growth factor and receptor mediated cell signaling process thus acting as a \( 2^{nd} \) messenger while, in higher or lethal doses it has a defensive role in cells by preventing them from microbe invasion through their killing.

2.1 (c) Hydrogen peroxide: Hydrogen peroxide (\( H_2O_2 \)) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states (Davies, 1999; Zhang, 2001). \( H_2O_2 \) functions through NFκ-B, hydroperoxide-mediated pathways and other factors. A recent report indicates that antibodies have the capacity to convert molecular oxygen into hydrogen peroxide to contribute to the normal recognition and destructive processes of immune system (Wentworth Jr., et. al.2001; Wentworth, et. al., 2000) measurement of this reactive intermediate help to determine macrophage activation.

2.1 (d) Lysozyme: Lysozyme which mediates digestion of bacterial cell walls, is a well-documented secretory product of macrophage. Unlike lysosomal hydrolases, which are largely retained within the macrophage and only released upon appropriate stimulation, lysozyme is secreted continuously. Furthermore, essentially all populations of macrophage produce and secrete large amounts of lysozyme (Gordon, 1974).

2.1 (e) NADH-NADPH: NADH and NAD+ are the reduced and oxidized forms, respectively, of the coenzyme NAD (Nelson, 2005). It is believed that the enhanced microbicidal and tumoricidal capability of activated macrophages is related to the remarkable increase in the production of oxygen metabolites. Both the production of \( H2O2 \) and the oxidation of NAD(P)H are directly dependent upon NAD(P)H-oxidase. It has been established that the respiratory burst is due to activation of NAD(P)H-oxidase localised in the plasmalemma (Dewald, 1979). Through receptor mediated interactions, extracellular
stimuli activate pathways that signal for the phosphorylation and assembly of the NADPH-oxidase. Once the NADPH-oxidase is activated, it produces superoxide and H2O2 in a process known as respiratory burst (Karen et al., 2002). The generation of reactive oxygen species (ROS) is associated with production of certain oxygen metabolites, which is linked to induction of cellular damage, microbicidal activity and the regulation of the activity of natural killer cells, polymorphonuclear leucocytes (PMNLs) and monocytes (Babior, 1984). The conversion of oxygen to microbicidal product is mediated by a plasma membrane-associated enzymes or enzyme system (Kumar et al., 2002).

2.1 (f) Myeloperoxidase: Myeloperoxidase (MPO; H2O2 oxidoreductase) is an enzyme found in the azurophilic granules of mammalian neutrophils and also identified in human monocytes. PMNLs employ a system comprised of MPO, H2O2 and oxidisable halide co-factor to kill a variety of microorganisms (Ding et al., 1988). MPO is believed to be involved in augmenting the cytotoxic activity of H2O2 and O2−. Therefore, NADH-oxidase, NADPH-oxidase and MPO activity were examined in macrophages treated with BRMs or LPS.

2.2 Materials and Methods:

2.2 (i) Reagents:
DMEM with L-glutamine and 25 mM HEPES buffer was purchased from (HiMedia Pvt. Ltd. India.), Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56°C for 45 min. Gentamicin (Ranbaxy Laboratories, Ltd. India) a sterile injectible antibiotic was commercially available in 80mg/2ml vials. The drugs used were obtained from Himalaya Drug Company products, India. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

2.2 (ii) Cells:
The macrophage J774A.1 cell line obtained from National Center for Cell Sciences [NCCS, Pune] used as source of macrophages, [Origin: BALB/c mouse; Nature: Mature] was grown and maintained in the DMEM enriched with 10% heat inactivated fetal Bovine serum, at 37°C and 5% CO2.
2.2 (iii) Viability Assay:
Cell viability was determined by the Trypan blue dye exclusion test. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for biochemical assay in $2 \times 10^6$ densities per ml in the 24 well tissue culture plates.

2.2 (iv) Stimulation of macrophages:
The macrophages from late log phase of growth [subconfluent] were seeded in 96 well plate or 24 well flat bottom microtiter plates [Tarsons, India] in a volume of 500µl under adequate culture conditions. Drugs were added in different concentrations in a volume of 500µl in triplicate. The cultures were incubated at 37ºC and 5% CO$_2$ environment. After 24h and 48h incubation cells were assayed for biochemical assays.

2.2 A] Assessment of Reactive nitrogen and oxygen intermediates:

2.2 A.1 Nitrite assay: The concentration of stable nitrite, an end product of the nitric oxide present in the supernatant of treated or untreated J774A.1 macrophage cell cultures ($2 \times 10^6$ cells/ml), was measured by the method of (Ding et al.,1988) based on the Griess reaction. Briefly, 50µl of supernatant was incubated with an equal volume of Griess reagent (1% sulphanilamide in 2.5% H$_3$PO$_4$ and 0.1% naphthyl-ethylene-diaminedihydrochloride in distilled water; both solutions mixed in a ratio of 1:1 at room temperature) for 10 min. The absorbance at 550 nm was then measured in a microtitre plate reader. The standard curve for nitrite was prepared by using 10-100 μM sodium nitrite in distilled water.

(fig source: activemotif.com)
2.2 A.2 Superoxide (O$_2^-$) assay:
Superoxide release was assayed by the method described by (Johnston et al., 1981). Briefly, 1.5 ml of reaction mixture containing 80 µM ferricytochrome C in HBSS without phenol red and PMA (500 ng/ml) was added to the 24h and 48h treated and untreated macrophage cultures (2x10$^6$ cells/ml) and the cultures incubated for 90 min in a CO$_2$ incubator at 37$^0$C. After 90 min, the reaction mixture was transferred to chilled centrifuge tubes and centrifuged at 1000 r.p.m. for 10 min at 4$^0$C. The absorbance was measured at 550 nm and production of O$_2^-$ calculated according to the formula:

nanomoles of O$_2^-$ released = 71.4 x OD$_{550}$.

2.2 A.3 Hydrogen peroxide H$_2$O$_2$ assay:
H$_2$O$_2$ release by treated and untreated macrophage cultures (2x10$^6$ cells/ml) was quantified according to the method described elsewhere (Pai and Sodhi, 1991) with slight modifications. Phenol red was dissolved in HBSS (without phenol red) at a concentration of 0.1 g/l. Purified horseradish peroxidase (HRP) was dissolved in HBSS at a concentration of 165U/ml. Macrophages were incubated in a CO$_2$ incubator for 1 h at 37$^0$C with the reaction mixture containing 5 ml of phenol red solution, 0.16 ml of HRP and 500 ng/ml PMA (final concentration). At the end of incubation, 0.1 N NaOH was added and absorbance was measured at 615nm. For the standard curve, H$_2$O$_2$ (10-100 µM) was prepared in quartz distilled water.


2.2 B.1 Assay for lysozyme:
Lysozyme assay was performed with culture supernatants from treated and untreated acrophage cultures by the method described by (Litwack, 1955). A substrate suspension of dried and killed Micrococcus luteus (0.1 mg/ml) was prepared in 0.1 M sodium phosphate buffer at pH 6.2 and 0.1 ml culture supernatant collected from treated and untreated macrophages were added to each tubes containing 2.5 ml of substrate buffer kept at 37$^0$C. Optical density at 600 nm was checked at zero hour of incubation. The tubes were then incubated at 37$^0$ C for 1 hr and decrease in optical density was checked at 600 nm. Experiments were repeated three times in triplicates and enzyme activity expressed in units/ml culture supernatant / 2 x 10$^6$ cells / ml.
2.2 B.2 NADH-NADPH assay:
The macrophage cells J774A.1 were treated with different drugs as Gallic acid, Guduchi, Spirulina, Canova, AOIM-Z, cisplatin and LPS. Cell supernatants were collected separately for other assays. Cells were washed with fresh culture medium and the cells were lysed in 2 ml of SDS 0.25% in distilled water; 0.5 ml of this cell lysate was added to a tube containing 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.5. The OD$_{340}$ of the sample was determined for NADPH- and NADH-oxidase activity. For preparing standards accurately weighed amounts of β-nicotinamide adenine dinucleotide(NADH) (2mg of each) were dissolved in 5ml of phosphate buffer, pH 7.5 The OD$_{340}$ of each of several dilutions of this stock solution was read against the buffer. A calibration curve was plotted and the OD$_{340}$ values of samples were compared with the standard OD$_{340}$ of the NADH-oxidase and NADPH-oxidase. The increase or decrease in the OD$_{340}$ was directly related to the increase or decrease in the enzyme activity. The data are represented as increase or decrease in OD$_{340}$. A calibration curve was plotted and the OD$_{340}$ values of samples were compared with the standard OD$_{340}$ of the NADH-oxidase and NADPH-oxidase. The increase or decrease in the OD$_{340}$ was directly related to the increase or decrease in the enzyme activity. The data are represented as increase or decrease in OD$_{340}$.

2.2 B.3 Assay for myeloperoxidase (MPO):
MPO activity of macrophages was assessed in the cell lysates by the method described elsewhere (5). Macrophages were lysed with Triton X-100 0.05% and were kept frozen at −70°C until use. The substrate cocktail contained 5 ml of 0.1 M citrate buffer (pH5.5), 32 μl of Triton X-100 20%, 50 μl of 82.4 mM Dianasidine or OPD in dimethyl sulphoxide (20.1 mg/ml in DMSO) and 20 μl of 26.4 mM H$_2$O$_2$. To 0.5 ml of cell lysate, 0.7 ml of 0.1 M citrate buffer (pH 5.5) was added. Then 4.8 ml of the above assay mixture was again added. The mixture was kept at room temperature for 1 h and the OD$_{450}$ was measured. Enzyme activity at room temperature = \( \frac{\text{ODe}_{450} \cdot \text{ODB}_{450}}{\text{Time(min)} \cdot \text{volume of samples (ml)}} \) units/min/ml of cell lysate

2.3 Statistical analysis:
Statistical significance of difference between the control and experimental samples were calculated by Student’s t-test. All the experiments were done in triplicate samples. Conclusions were drawn from 3 independent experiments.
2.4 Results:

2.4 A) Assessment of reactive nitrogen and oxygen species:

2.4 A.1 Nitrite assay: J774A.1 cells were incubated in medium alone or drugs for 24h and 48h and the cell supernatants were checked for nitrite. Cell supernatants of macrophages treated with drugs at log and linear scales for 24h and 48h were collected during cytotoxicity assay (chapter1) and were examined for nitrite assay. It showed significantly increased levels of nitrite as compared to cells treated with medium alone. Similar results were obtained with the LPS treated cell supernatant. According to the cytotoxicity assay described in chapter1, the drug concentrations where maximum viability of macrophages was observed also showed enhanced nitrite levels at log scale. Supernatants of macrophages treated with the optimum drug concentrations for 24h and 48h, showed significantly enhanced nitrite levels as compared to supernatants collected from macrophages treated with medium alone. Macrophages on treatment with the herbal drugs guduchi and spirulina showed most enhanced nitrite levels in comparison with other drug treatments and also were compared with the results of standard positive control (LPS) treated macrophage cell supernatant.

2.4 A.2 Superoxide assay:

Macrophage cells J774A.1 (2x10^6) incubated in medium alone produced 6.6±0.05, 0.54±0.05nM of superoxide (O_2^-) anions after 24h and 48h respectively. Cells treated with guduchi, AOIM-Z and LPS produced significantly higher amounts of O_2^- (8.6±0.5, 3.2±0.01; 8.2±0.05, 1.3±0.01 and 8±0.5, 4±1) after 24h and 48h treatment respectively. Canova and cisplatin treated macrophages also showed enhanced superoxide levels (7.9±0.05, 0.7±0.01 and 7.5±0.06, 4±0.1nM) after 24 and 48h treatment respectively. It was observed that 48h treatment of drug for macrophages was most effective resulting in the release of O_2^-. After 48h treatment the gallic acid and spirulina treated cells showed increased O_2^- levels (6.67±0.1, 4.3±0.5 and 6.61±0.05, 1.4±0.05nM, respectively) when compared to cells treated with medium alone (Table6).
Fig 16: Results for effect of log scale concentrations of drugs on nitrite levels of J774A.1 cells:

**a) gallic acid**

![Nitrite levels (Gallic acid)](image)

**b) guduchi**

![Nitrite levels (Guduchi)](image)

**c) canova**

![Nitrite levels (Canova)](image)

**d) spirulina**

![Nitrite levels (Spirulina)](image)

**e) AOIM-Z**

![Nitrite levels (AOIM-Z)](image)

**f) cisplatin**

![Nitrite levels (Cisplatin)](image)

**g) LPS**

![Nitrite levels (LPS)](image)

Fig 16: The culture supernatants were collected from macrophages treated with LPS, gallic acid, guduchi, cisplatin, spirulina, AOIM-Z and canova and checked for generation of nitrite. Values represent nitrite contents in µM. Bars shows SDs of means for triplicate cultures. Data are representative of three separate experiments. (*p<0.05).
**Fig 17**: Results for effect of linear scale concentrations of drugs on nitrite levels of J774A.1 cells

![Graph showing nitrite levels](image)

**Fig 17**: The culture supernatants were collected from treated and untreated macrophages with LPS, Gallic acid derivative of *Emblica officinalis*, guduchi, cisplatin, spirulina, AOIM-Z and canova and checked for generation of Nitrite. Values represent Nitrite content in µM. Bars shows SDs of means for triplicate (cultures. Data are representative of three separate experiments. (*p<0.05, **p<0.001).

**Table 6**: Ferricytochrome C reduction in nanomoles/2x10^6 cells/ml in presence of PMA from drug treated and untreated macrophage cells (J774A.1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Superoxide levels (nm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Concentration (µg/ml)</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>60</td>
</tr>
<tr>
<td>Guduchi</td>
<td>80</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10</td>
</tr>
<tr>
<td>Canova</td>
<td>0.1c</td>
</tr>
<tr>
<td>Spirulina</td>
<td>100</td>
</tr>
<tr>
<td>AOIM-Z</td>
<td>80</td>
</tr>
<tr>
<td>LPS</td>
<td>10</td>
</tr>
<tr>
<td>Medium alone</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6**: The values given in the table are mean± standard deviation and represent three independent experiments done in triplicate. *significantly increased versus untreated controls (*p<0.01).
Table 7: Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for hydrogen peroxide ($H_2O_2$) release.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrogen peroxide release (nM/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Concentration (µg/ml) 24h 48h</td>
</tr>
<tr>
<td>Guduchi</td>
<td>80                  16.36±0.5 33.91±0.01*</td>
</tr>
<tr>
<td>LPS</td>
<td>10                  15.65±0.5* 43.23±1*</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>60                  15.52±0.01* 31.10±0.05</td>
</tr>
<tr>
<td>Canova/Anbuta</td>
<td>0.1                 13.47±0.5 29.70±0.05</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10                  14.83±0.02* 31.07±0.05</td>
</tr>
<tr>
<td>AOIM-Z</td>
<td>80                  16.44±0.05* 30.79±0.5</td>
</tr>
<tr>
<td>Spirulina</td>
<td>100                 13.40±0.5 31.87±0.5</td>
</tr>
<tr>
<td>Medium alone</td>
<td>12.42±0.05 29.64±0.05</td>
</tr>
</tbody>
</table>

Table 7: The values are mean ±S.D. and representative of three different experiments done in triplicate. *P< 0.05; significantly different from respective controls.

2.4 A.3 $H_2O_2$ release

A significant increase in $H_2O_2$ (in nanomoles/2x10^6 macrophages/ml) was observed in macrophages treated with BRMs and LPS. And the results were compared with cells treated with medium alone. Guduchi treated macrophages showed a significant increase in $H_2O_2$ from 16.2 to 33.91nM/ml for 24h and 48h of treatment. Whereas for macrophages treated with medium alone the yield of $H_2O_2$ remained low (12.42±0.05 and 29.64±0.05nM/ml) after 24 and 48h, respectively (Table 7). Gallic acid, AOIM-Z and cisplatin treated macrophages also showed enhanced $H_2O_2$ production (15.52±0.01, 31.10±0.05; 16.44±0.05, 30.79±0.5 and 14.83±0.02, 31.07±0.05nM/ml) after 24h and 48h, respectively. Spirulina treated macrophages have also showed enhanced $H_2O_2$ levels after 48h treatment. The results were comparable with the $H_2O_2$ levels produced by macrophages treated with the standard positive control LPS (15.65±0.5, 43.23±1nM/ml) after 24h and 48h, respectively (Table 7).

2.4 B.1 Secretion of lysozyme:

Macrophages treated with BRMs showed increased release of lysozyme as compared to untreated cells as assessed by supernatants. Decreased turbidity of *Micrococcus luteus* in the substrate buffer (as a measure of lysozyme) was observed after treatment
with the supernatants of drug treated cells. A significant level of lysozyme was observed in the supernatants after 24h and 48h of treatment. The lysozyme levels were measured in units/ml of culture supernatants/2 x10^6 cells/ml. Canova and AOIM-Z treatment showed enhanced lysozyme levels especially after 48h treatment (Fig18). Gallic acid, guduchi and cisplatin treated macrophages also showed significantly increased lysozyme levels after 24 and 48h treatment (Fig18, Table8).

**Fig18:** Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for lysozyme release.

**Fig18:** Enhanced lysozyme levels were observed as compared to controls (Units/ml of culture supernatant/2 x106 cells/ml. Absorbance measured at 600nm wavelength). (*p<0.05, **p<0.001).

**Table8:** The culture supernatants were collected from treated and untreated macrophages with LPS or BRMs and checked for lysozyme activity. Values represent enzyme activity in units/ml of culture supernatant/2*10⁶ cells/ml

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lysozyme levels (Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>Medium alone</td>
<td>1.1 ±0.1</td>
</tr>
<tr>
<td>Cisplatin (10µg/ml)</td>
<td>2.5 ±0.11</td>
</tr>
<tr>
<td>Gallic acid(60µg/ml)</td>
<td>1.7 ±0.05*</td>
</tr>
<tr>
<td>LPS (10µg/ml)</td>
<td>2.5 ±0.25</td>
</tr>
<tr>
<td>Guduchi (80µg/ml)</td>
<td>2.2 ±0.1 **</td>
</tr>
<tr>
<td>Spirulina(100µg/ml)</td>
<td>1.2±0.1*</td>
</tr>
<tr>
<td>AOIM-Z (80µg/ml)</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Canova (0.1c/ml)</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>

**Table8:** Enhanced lysozyme levels were observed as compared to controls (Units/ml of culture supernatant/2 x10⁶ cells /ml. Absorbance measured at 600nm wavelength) (*p<0.05, **p<0.001).
2.4 B.2 NADH-oxidase activity in macrophages:
Macrophage lysates were assayed for their NADH oxidase activity against the standards. The increase or decrease in OD$_{340}$ directly shows increased or decreased NADH-oxidase activity. Macrophage cells after guduchi, spirulina and canova treatment showed maximum and significantly greater NADH-oxidase activity [(316±5.7, 310±4.8); (265±5.8, 245±5.8) and (318±6, 298±5) (OD$_{340}$); after 24h and 48h, respectively, as compared to untreated controls [83±4.1 and 79±4 (OD$_{340}$)] after 24h and 48h, respectively. Cisplatin and AOIM-Z [132±4.1 and 120±4 (OD$_{340}$); 24h and 48h, respectively] treatment to macrophages also enhanced the NADH-oxidase activity. The conclusions were drawn by comparing these results with the NADH-oxidase activity after LPS treatment [233±5 and 228±8 (OD$_{340}$); after 24h and 48h treatment, respectively] (Figure 20).

2.4 B.3 NADPH-oxidase activity in macrophages:
Macrophage lysates were assayed for their NADPH-oxidase activity against the standards. The increase or decrease in OD$_{340}$ directly shows increased or decreased NADPH-oxidase activity. Macrophage after guduchi, spirulina, canova and AOIM-Z treatment showed significantly greater NADPH-oxidase activity [(234±4, 231±4.2); (260±2, 245±4); (219±1,191±1) and (155±2,157±2) (OD$_{340}$); 24h and 48h, respectively] than those from untreated controls [(25±3 and 21±0.6) (OD$_{340}$); 24h and 48h, respectively]. Gallic acid and cisplatin treatment also enhanced NADPH-oxidase activity [(121±1,117±2) and (74±3, 68±5) after 24h and 48h, respectively] the results were compared with the NADPH-oxidase activity of LPS treated macrophages [(238±8 and 220±4 (OD$_{340}$); after 24h and 48h treatment, respectively)] (Figure 21).

2.4 B.4 Myeloperoxidase (MPO) activity in macrophages:
Macrophage lysates from drug treated and untreated cells were assayed for their myeloperoxidase (MPO) activity. It was observed that guduchi, spirulina and AOIM-Z treated macrophages (J774A.1) showed [(7.8 × 10$^{-4}$ ± 0.3, 6.45 × 10$^{-4}$ ± 0.5 U/min/ml); (8.54 × 10$^{-4}$ ±0.3, 7.23 × 10$^{-4}$ ±0.3 U/min/ml) and (9.36 × 10$^{-4}$ ±0.3, 9.1 x 10$^{-4}$ ±0.3 U/min/ml)] MPO activity, after 24h and 48h, respectively. Also macrophages treated with LPS showed (5.96 × 10$^{-4}$ ± 0.5 U/min/ml and 5.92 × 10$^{-4}$± 0.15 U/min/ml) MPO activity, after 24h and 48h, respectively. The
myeloperoxidase activity of drug treated cells was found to be significantly higher than those from untreated controls (4.55 × 10⁻⁴ ± 0.25 and 4.2 × 10⁻⁴ ± 0.5) (Table 9). Gallic acid, canova and cisplatin treated macrophages also showed enhancement in MPO activity [(6.64 x 10⁻⁴ ±0.13, 5.43 x 10⁻⁴ ±0.11 U/min/ml); (6.07 x 10⁻⁴ ±0.2, 5.83 x 10⁻⁴ ±0.3 U/min/ml) and (5.44 x 10⁻⁴ ±0.25, 3.91 x 10⁻⁴ ±0.1 U/min/ml)] after 24h and 48h, respectively.

Fig19: Standard NADH-NADPH oxidase.

Fig 20 and 21: Effect of BRMs on the NADH and NADPH oxidase activity of macrophages.

Fig 20: In-vitro NADH-oxidase activity (µg/2x10⁶ cells/ml) in drug treated and untreated macrophages (J774A.1).

Fig20: Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for NADH-oxidase activity. The values are mean S.D. and representative of three independent experiments done in triplicate. *P< 0.00003; significantly different from respective controls.
Fig 21: In-vitro NADPH-oxidase activity (µg/2x10^6 cells/ml) in drug treated and untreated macrophages (J774A.1).

Table 9: Myeloperoxidase activity in macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPO (U/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>Medium alone</td>
<td>4.55 x 10^{-4} ±0.25</td>
</tr>
<tr>
<td>Guduchi (80 µg/ml)</td>
<td>7.8 x 10^{-4} ±0.3*</td>
</tr>
<tr>
<td>Gallic acid (60 µg/ml)</td>
<td>6.64 x 10^{-4} ±0.13*</td>
</tr>
<tr>
<td>Canova (0.1c /ml)</td>
<td>6.07 x 10^{-4} ±0.2*</td>
</tr>
<tr>
<td>Spirulina (100 µg/ml)</td>
<td>8.54 x 10^{-4} ±0.3*</td>
</tr>
<tr>
<td>AOIM-Z (80 µg/ml)</td>
<td>9.36 x 10^{-4} ±0.3*</td>
</tr>
<tr>
<td>Cisplatin (10 µg/ml)</td>
<td>5.44 x 10^{-4} ±0.25</td>
</tr>
<tr>
<td>LPS (10 µg/ml)</td>
<td>5.96 x 10^{-4} ±0.5</td>
</tr>
</tbody>
</table>

Table 9: Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for myeloperoxidase activity. The values are mean S.D. and representative of three independent experiments done in triplicate. *P< 0.03; significantly different from respective controls.
CHAPTER 2  EVALUATION OF BIOCHEMICAL PARAMETERS OF MACROPHAGE ACTIVATION

2.5 Discussion

Macrophage activation is being better understood with the biochemical and cytochemical assays. In actively respiring cells, superoxide and hydrogen peroxide can be activated which further generates reactive oxygen species (ROS). Macrophages are dynamic cells which are the first line of defense and constitute an important participant in the bi-directional interaction between innate and specific immunity. Our environment contains a great variety of infectious microbes that may be potentially destructive and threaten our survival. As soon as microbes try to establish a site of infection, the host launches a complex defense mechanism. Innate immunity is a non-specific response and serves as the first line of defense where phagocytes, such as neutrophils, macrophages, and NK cells play central roles in neutralizing and clearing microorganisms. Thus, migration of cells into infectious foci and subsequent activation of these cells appear to be a critical step, enabling the host to achieve effective and efficient removal of microbes (Kumar et al., 2001). The present study addresses the immunomodulatory effects of BRMs on macrophage activation.

It is believed that the enhanced capability of activated macrophages to resist infection (Winterbourn et al., 2000) is related to the remarkable increase in the production of oxygen metabolites in response to phagocytosis. It is further believed that the greater oxidative metabolic responses of the activated macrophages could play a marked role in the accelerated capacity to affect cell-mediated immunity. In the present study, increased NADH-oxidase and NADPH-oxidase activity was observed in macrophages treated with BRMs or LPS as compared to macrophages treated with medium alone. Our findings showed that BRMs or LPS treated macrophages significantly enhanced the NADH- and NADPH-oxidase (Figures 20 and 21) as compared to macrophages treated with medium alone. It also significantly enhanced MPO levels (Table 9) after drug treatment for 24 h and 48 h, respectively. It is suggested that up-regulated activity of NADH-oxidase and NADPH-oxidase implicate activated state of macrophages which further augments the microbicidal and tumoricidal functions (Mackeness, 1970). As ROIs, a result of respiratory burst activity, are essential for microbicidal and tumoricidal activity of macrophages, both the production of H₂O₂ and oxidation of NADPH are directly dependent upon NADPH-oxidase. The results
of the study indicates that BRM treated macrophages (J774A.1) showed enhanced levels of reactive nitrogen and oxygen species i.e. nitrite, hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$). Also enhanced enzyme levels (lysozyme, NADH-NADPH and myeloperoxidase) were found by cultured macrophages which were significantly higher than the macrophages treated with medium alone. Thus, enhanced production of nitrite, lysozyme, H$_2$O$_2$, O$_2^-$ suggests that up-regulation of these mediators might be involved in the killing of microbes by macrophages treated with BRMs.

Furthermore, it is suggested that an immune-based mechanism is involved in successful drug therapy (Burner et. al., 2000). It has been established that the respiratory burst is due to activation of NAD(P)H-oxidase localised in the plasmalemma. Intracellular oxygen-dependent bactericidal toxicity is mediated among other molecules through myeloperoxidase, which catalyzes the oxidative reaction of the peroxide substrates and consequently leads to the increase in the radical intermediates in the cellular environment (Beutler, 2004; Hackette, 2003; Lolis and Bucala, 2003; Tzianabos, 2000). Being present in cytoplasmic granules of neutrophils and monocytes, MPO participates in the early inflammatory process and affects further course of the numerous diseases (Singh et.al., 2006; Klebanoff, 1999). The enzyme is also released to the outside of the phagocytic cells in the extracellular medium where induction of macrophages for some cytokines production was observed (Van Dalen, 2000) and where MPO measurement can reflect their activation (Lolis and Bucala, 2003).

2.6 Conclusion

On stimulation with appropriate agents in vitro the macrophages get activated and result in enhanced production of reactive nitrogen and oxygen intermediates and different enzymes. We conclude that there is an up-regulation of NADH-oxidase, NADPH-oxidase and MPO (biochemical parameters of macrophage activation) activity in macrophages (J774A.1) after BRMs treatment. The results of present study show experimental basis of immunomodulation by biological response modifiers (BRMs). This study addresses a very pertinent question of biomedical sciences dealing with the scientific basis, particularly immunomodulatory effects, of the herbal medicine preparations on the macrophage activation, as macrophages are known to represent the first line of defense against invading microorganisms or in a state of altered self.