The Therapeutic effect of MKA on Bacterial Lipopolysaccharide (LPS) induced lipid peroxidation, cytosolic LDH leakage and mitochondrial membrane depolarization in RAW 264.7 Macrophages

Poongodi T\textsuperscript{1}, Nazeema T H\textsuperscript{2}

\textsuperscript{1}Department of Biochemistry, Rathnavel Subramaniam College of Arts and Science, Coimbatore-641402, Tamil Nadu, India
\textsuperscript{2}Department of Life Sciences, Michael Job College of Arts and Science for Women, Coimbatore-641103, Tamil Nadu, India

\textbf{Article History:}
Received on: 15 Aug 2020
Revised on: 16 Sep 2020
Accepted on: 17 Sep 2020

\textbf{Keywords:}
Malondialdehyde, Inflammation, Flow cytometry, depolarisation

\textbf{ABSTRACT}

The Polyherbal formulations are used as a potential target for treating various diseases due to its wide array of phytoconstituents with antioxidant potential. In the present study, the therapeutic effect of Polyherbal formulation (MKA) comprising of three plants \textit{Mimusops elengi} L., \textit{Kedrostis foetidissima} (Jacq.) Cogn. and \textit{Artemisia vulgaris} L. were studied in LPS induced RAW 264.7 macrophages. Four different concentrations (25, 50, 75 and 100 \(\mu\)g/ml) of MKA were tested against control, LPS treatment and standard Quercetin in LPS induced RAW 264.7 macrophage cells. The rate of Lipid peroxidation was measured in terms of Malondialdehyde (MDA) levels. The cytosolic LDH leakage was determined by measuring NADH release at 340nm. The changes in mitochondrial membrane potential were studied by measuring red/green fluorescent intensity of JC-1 stained cells in the flow cytometer. It was found that MKA treatments significantly reduced the rate of Lipid peroxidation and LDH leakage compared to LPS treatment. The results of flow cytometry revealed that the JC-1 green fluorescent intensity decreased with increase in MKA concentration, in a dose-dependent manner. It is evident from the study results that, the MKA has a therapeutic effect on LPS induced RAW 264.7 macrophages by protecting the cells from lipid peroxidation, restoring the cell membrane integrity and mitochondrial membrane potential.

\*Corresponding Author
Name: Poongodi T
Phone: +91-9600694197
Email: poongodi.btw@gmail.com

ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11i4.3306](https://doi.org/10.26452/ijrps.v11i4.3306)

\section*{INTRODUCTION}

Reactive oxygen species (ROS) is the main causative agent of diseases like cardiovascular diseases, cancer, pulmonary diseases, atherosclerosis and inflammatory diseases. Oxidative stress occurs in a biological system as a result of an improper balance between formation and neutralization of ROS (Fridovich, 1999; Fang \textit{et al.}, 2002). Antioxidants can detoxify ROS by acting as radical scavengers, electron donors, singlet oxygen quenchers, synergists, hydrogen donors, metal-chelating agents, peroxide decomposers and enzyme inhibitors (Frei \textit{et al.}, 1988). Herbal medicines are a rich source of phytochemicals, which acts as potent antioxidants with free radical scavenging activity. The synergistic action of Polyherbal formulations renders a more therapeutic effect compared to single herbal preparation (Parasuraman \textit{et al.}, 2014).
The Lipopolysaccharide (LPS) is a bacterial endotoxin, which functions as a potent activator of RAW 264.7 macrophages and triggers the release of inflammatory mediators leading to acute and chronic inflammatory conditions (Veres et al., 2014; Troutman et al., 2012). There is an increase in the level of ROS and lipid peroxidation rate in LPS triggered macrophages (Ambrosova et al., 2011).

Excess of ROS production affects the function of Calcium (Ca$^{2+}$) regulating proteins and other electron-transport chain proteins in mitochondria, thereby altering the mitochondrial membrane potential (Guo et al., 2013).

The ROS acts on membrane lipids inducing lipid peroxidation which releases toxic lipid-derived aldehydes (LDAs) like malondialdehyde (MDA), acrolein and 4-hydroxy-trans-2-nonenal (HNE). The LDAs activate various kinases involved in redox signaling pathways leading to the cytotoxicity of the cell, ultimately causing cell death (Yadav, 2015). The excess of LDH leakage from the cytoplasm into the extracellular portion is indicative of the extent of cell membrane damage (Li et al., 2014).

In the present investigation, the protective action of MKA, the polyherbal formulation comprising of three selected plants *Mimusops elengi* (L.), *Kedrostis foetidissima* (Jacq.) Cogn. and *Artemisia vulgaris* (L.) was studied in LPS primed macrophage cells. The lipid peroxidation rate, cytosolic LDH leakage, changes in mitochondrial membrane potential were analyzed in LPS induced RAW 264.7 macrophages upon treatment with different concentrations of MKA. This study is the continuation of the previous research entitled “Evaluation of free radical scavenging capacity and reducing the power of polyherbal formulation comprising of three selected plants” (Poongodi and Nazeema, 2019).

**MATERIALS AND METHODS**

**Sampling and Preparation of Polyherbal formulation**

The leaf sample of three plants *Mimusops elengi* (L.), *Kedrostis foetidissima* (Jacq.) Cogn. and *Artemisia vulgaris* (L.) were collected from Sulur, Coimbatore. The plants were authenticated from Botanical Survey of India (BSI), TNAU, Coimbatore. The authentication reference number for *Mimusops elengi* (L) is BSI/SRC/5/23/2018/Tech/1224, *Kedrostis foetidissima* (Jacq.) Cogn. are BSI/SRC/5/23/2018/Tech/1225, and *Artemisia vulgaris* (L.) is BSI/SRC/5/23/2018/Tech/1226 (Poongodi and Nazeema, 2019).

The Polyherbal formulation MKA was prepared using a leaf sample of three plants *Mimusops elengi* (L.), *Kedrostis foetidissima* (Jacq.) Cogn. and *Artemisia vulgaris* (L.) in ratio 1:1:1. The sample is extracted for phytoconstituents using 80% ethanol in Soxhlet apparatus. The obtained extract was evaporated in rotavaporator, and the resultant sample is dissolved in DMSO and stored for further studies (Poongodi and Nazeema, 2019).

**RAW 264.7 Macrophage Cell Culture**

RAW 264.7 macrophage cell line was purchased from NCCS, Pune. The macrophage cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO$_2$. The cultured macrophage cells were washed in DMEM and detached the cells using 0.25% trypsin-EDTA solution (Lee and Park, 2011).

**Cell treatment**

The cell treatment was performed by the method of Khan et al. (1995) modified method. The macrophage cells were seeded in 6-well plate at a density of 5x10$^3$ cells per well and incubated for 24 hours at 37°C in 5% CO$_2$. The cells were washed with DMEM solution and added 1600µl of growth medium. Cells were treated with 200µl of MKA at four different concentrations 25, 50, 75 and 100 µg/ml and incubated for 1-2 hours before LPS treatment. Quercetin was used as standard at a concentration of 25µM. Then, added LPS (1µg/ml) and incubated for 24 hours at 37°C in 5% CO$_2$. The negative control (without LPS and MKA) and Positive LPS (without MKA) were also studied. All these mixtures were centrifuged at 2000xg for 10 minutes. The supernatant was used to study Lipid peroxidation and LDH leakage. The pelleted cells were used to study mitochondrial membrane potential.

**Lipid Peroxidation**

The lipid peroxidation was studied by the method of Draper and Hadley (1990). The culture supernatants were used to study the malondialdehyde (MDA) levels, which is an indicator of ROS mediated lipid peroxidation. To 0.5 ml of media supernatant, added 1ml of 30% trichloroacetic acid (TCA) and centrifuged at 3500xg for 10 minutes. Then, 1 ml of this supernatant was mixed with 1 ml of thiobarbituric acid (TBA) and heated this mixture at 90°C for 10 minutes and cooled. The MDA levels were measured at 532 nm. The standard curve was plotted using 1,1,3,3 tetra ethoxy propane. The results are expressed as nanomoles of MDA equivalents.

**Cell Membrane integrity assay (LDH leakage)**

Membrane integrity was studied in terms of extra-

cellular LDH leakage. The LDH in the cell supernatant was evaluated by monitoring the decrease in the level of NADH at 340nm in a microtitre plate reader (Anthos 2020, Austria). This decrease in NADH level is due to the conversion of pyruvate to lactate by LDH. The LDH leakage is directly proportional to the level of membrane damage (Pereira et al., 2015).

Flow cytometric analysis of mitochondrial membrane potential

The pelleted cells were incubated in media containing one µg/ml of JC-1 dye for 15 minutes at 37°C in a CO₂ incubator. Then the cells were washed twice with phenol-red free media to remove the unbound dye. The mitochondrial membrane potential was immediately studied as the ratio of red and green fluorescence in Flow cytometer (BD FACSverse) (Venkatesan et al., 2017).

Statistical analysis

All experiments were performed in triplicates, and the results were expressed as Mean±Standard Deviations (SD). Data were analyzed using one way ANOVA followed by post hoc Dunnett’s multiple comparison test using SPSS software (Version 21). P<0.01 were considered statistically significant.

RESULTS AND DISCUSSION

The effect of MKA on Lipid Peroxidation in RAW 264.7 macrophages

Lipid peroxidation was measured in terms of MDA levels, which is elevated in conditions of oxidative stress. In Figure 1, the effect of MKA on the rate of lipid peroxidation in LPS induced RAW 264.7 cells were illustrated. In LPS treated macrophages, MDA level was found to be 2.86±0.13 nM. In 25, 50, 75 and 100 µg/ml of MKA treatments, MDA levels reduced to 2.23±0.15, 1.45±0.24, 0.91±0.01, 0.73±0.05 nM respectively. The MDA level was 0.62±0.02 nM/mg in Quercetin treatment. Data are presented as Mean±SD of three replications; **represent significant difference (P<0.01) vs LPS; # represent significant difference (P<0.01) vs Control. Reactive oxygen species (ROS) produced in the normal physiological process has an essential role in tissue homeostasis and cell signalling pathways. Excess ROS production damages the cell membrane by lipid peroxidation of membrane lipids (Ferreira et al., 2018; Lundgren et al., 2018; Que et al., 2018).

The polyherbal formulation MKA comprising of Mimusops elengi L., Kedrostis foetidissima (Jacq.) Cogn. and Artemisia vulgaris L. reduced the level of lipid peroxidation induced by bacterial endotoxin in LPS macrophages. In LPS treated macrophages, MDA level was found to be significantly higher compared to control, which is an indication of oxidative stress due to lipid peroxidation. In 25, 50, 75 and 100 µg/ml of MKA treatments, MDA level significantly reduced compared to LPS treatment. MKA has therapeutic potential which can reduce lipid peroxidation in a dose-dependent manner, thereby preventing further cell and organelle damage.

LPS Induced LDH Leakage in Cell Supernatant (Membrane integrity Assay)

LPS induced inflammation causes cell membrane damage, resulting in leakage of Lactate dehydrogenase enzyme. So, the measurement of LDH leakage is an indicator of the extent of inflammation in LPS induced RAW 264.7 cells. LDH leakage levels were measured in cell supernatant and illustrated in Table 1. Significant increase of 844.97±25.39 U/L of LDH was seen in LPS treated cells compared to control. Also, it was evident that MKA treatment significantly reduced LDH leakage. LDH release in 100 µg/ml of MKA treatment was 309.92±19.75 U/L. In Quercetin treatment, LDH release was found to be 298.86±11.99 U/L. This confirmed that MKA reduced the inflammatory damage, thereby reducing the LDH release from LPS induced RAW 264.7 cells.

The effect of MKA on cell membrane integrity was studied in terms of cytosolic LDH leakage. The study results revealed that MKA has a protective effect on the cell membrane, reducing LDH leakage significantly compared to LPS treatment.

The effect of MKA on LPS induced mitochondrial membrane depolarisation

LPS induction caused mitochondrial membrane damage in RAW 264.7 macrophages, which was studied in terms of mitochondrial membrane poten-
Figure 2: Flow cytometry analysis of mitochondrial membrane potential using JC-1 staining.

Figure 3: Mitochondrial membrane potential represented as JC-1 green fluorescent intensity.

Table 1: Lactate dehydrogenase leakage

<table>
<thead>
<tr>
<th>Concentration</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>280.64 ± 14.32</td>
</tr>
<tr>
<td>LPS</td>
<td>844.97 ± 25.39#</td>
</tr>
<tr>
<td>MKA(25 µg/ml) + LPS</td>
<td>714.82 ± 16.01**</td>
</tr>
<tr>
<td>MKA(50 µg/ml) + LPS</td>
<td>648.32 ± 12.27**</td>
</tr>
<tr>
<td>MKA(75 µg/ml) + LPS</td>
<td>346.47 ± 20.75**</td>
</tr>
<tr>
<td>MKA(100 µg/ml) + LPS</td>
<td>309.92 ± 19.75**</td>
</tr>
<tr>
<td>Standard + LPS</td>
<td>298.86 ± 11.99**</td>
</tr>
</tbody>
</table>

The effect of MKA on mitochondrial depolarization of LPS induced RAW 264.7 macrophages were illustrated in Figure 2, (1) Control without MKA and LPS; (2) LPS treatment without MKA; (3) MKA (25 µg/ml) + LPS treatment; (4) MKA (50 µg/ml) + LPS treatment; (5) MKA (75 µg/ml) + LPS treatment; (6) MKA (100 µg/ml) + LPS treatment and (7) Quercetin standard (25 µM) + LPS treatment. The JC-1 green fluorescence intensity is enhanced in conditions of mitochondrial membrane damage due to membrane depolarization. In Figure 3, the mitochondrial membrane potential is represented in terms of green fluorescence intensity. ** represent significant difference (P<0.01) vs LPS; ## represent significant difference (P<0.01) vs Control; # represent no significant difference (P<0.01) among the group.

It was found that there is a significant increase (p<0.01) in the mitochondrial membrane potential...
of LPS treated compared to control. Also, there is a significant decrease (p<0.01) in the mitochondrial membrane potential of MKA treatments 25, 50, 75 and 100 μg/ml compared to LPS treatment. It was also noted that there is no significant difference between 100 μg/ml MKA treatment and standard Quercetin. The MKA has therapeutic property in restoring the mitochondrial membrane damage caused by LPS in macrophages. The LPS induction shifts the fluorescence signal to more green, indicating the high level of mitochondrial membrane depolarisation. The MKA treatment shifted the fluorescent signal to normal, thereby reducing the membrane depolarisation and restoring the mitochondrial membrane damage in a dose-dependent manner.

CONCLUSION

The Polyherbal formulation MKA, reduced the rate of lipid peroxidation in LPS triggered cells, which proves its efficacy in protecting the cells from oxidative damage. The LDH leakage was also reduced in MKA treatments, which validates its potential in protecting the cell membrane from oxidative damage. Also, the MKA restores the mitochondrial membrane potential significantly compared to LPS treated group. From these study results, it is evident that the MKA has a therapeutic role in protecting the macrophages from LPS induced oxidative stress.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for providing laboratory facility for this research work.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

Funding Support

The authors declare that they have no funding support for this study.

REFERENCES


