ORIGINAL ARTICLE

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

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**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 *Mimusops elengi* L., *Kedrostis foetidissima* (Jacq.) Cogn. and *Artemisia vulgaris* L. were studied in LPS induced RAW 264.7 macrophage cells. Four different concentrations (25, 50, 75 and 100 µg/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.

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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., 2004; Troutman et al., 2012). There is an increase in the level of ROS and lipid peroxidation rate in LPS triggered macrophages (Ambrozova et al., 2011).

Excess of ROS production affects the function of Calcium (Ca²⁺) 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 signalling 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 plants were authenticated from Botanical Survey of India (BSI), TNAU, Coimbatore. The plants were authenticated from Botanical Survey of India (BSI), TNAU, Coimbatore. The samples are dissolved in DMSO and stored for further studies (Poongodi and Nazeema, 2019).

ThePolyherbal 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% CO₂. 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 5x10⁶ cells per well and incubated for 24 hours at 37°C in 5% CO₂. 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% CO₂. 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 pellet 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 in Quercetin treatment was 0.62±0.02 nM/mg. 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

| Concentration                  | LDH (U/L)          |
|--------------------------------|--------------------|
| Control                        | 280.64 ± 14.32     |
| LPS                            | 844.97 ± 25.39#    |
| MKA (25 μg/ml) + LPS           | 714.82 ± 16.01**   |
| MKA (50 μg/ml) + LPS           | 648.32 ± 12.27**   |
| MKA (75 μg/ml) + LPS           | 346.47 ± 20.75**   |
| MKA (100 μg/ml) + LPS          | 309.92 ± 19.75**   |
| Standard + LPS                 | 298.86 ± 11.99**   |

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 fluorescent 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

Ambrozova, G., Pekarova, M., Lojek, A. 2011. The effect of lipid peroxidation products on reactive oxygen species formation and nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophages. Toxicology in Vitro, 25(1):145–152.

Draper, H. H., Hadley, M. 1990. Malondialdehyde determination as index of lipid Peroxidation. Methods in Enzymology 186, pages 421–431.

Fang, Y. Z., Yang, S., Wu, G. 2002. Free radicals, antioxidants, and nutrition. Nutrition, 18(10):872–879.

Ferreira, C. A., Ni, D., Rosenkrans, Z. T., Cai, W. 2018. Scavenging of reactive oxygen and nitrogen species with nanomaterials. Nano Research, 11(10):4955–4984.

Frei, B., Stocker, R., Ames, B. N. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. Proceedings of the National Academy of Sciences, 85(24):9748–9752.

Fridovich, I. 1999. Fundamental Aspects of Reactive Oxygen Species, or What’s the Matter with Oxygen? Annals of the New York Academy of Sciences, 893(1):13–18.

Galley, H. F. 2011. Oxidative stress and mitochondrial dysfunction in sepsis. British Journal of Anaesthesia, 107(1):57–64.

Guo, C., Sun, L., Chen, X., Zhang, D. 2013. Oxidative stress, mitochondrial damage and neurodegenerative diseases. Neural regeneration research, 8(21):2003–2014.

Khan, T. Z., Wagener, J. S., Bost, T., Martinez, J., Accurso, F. J., Riches, D. W. 1995. Early pulmonary inflammation in infants with cystic fibrosis. American Journal of Respiratory and Critical Care Medicine, 151(4):1075–1082.

Lee, J. Y., Park, W. 2011. Anti-Inflammatory Effect of Myristicin on RAW 264.7 Macrophages Stimulated with Polyinosinic-Polyribosyl Acid. Molecules, 16(8):7132–7142.

Li, X., Xiao, Y., Cui, Y., Tan, T., Narasimhulu, C. A., Hao, H., Liu, L., Zhang, J., He, G., Verfaillie, C. M., Lei, M., Parthasarathy, S., Ma, J., Zhu, H., Liu, Z. 2014. Cell membrane damage is involved in the impaired survival of bone marrow stem cells by oxidized low-density lipoprotein. Journal of Cellular and Molecular Medicine, 18(12):2445–2453.

Lundgren, C. A. K., Sjöstrand, D., Biner, O., Bennett, M., Rudling, A., Johansson, A.-L., Brzezinski, P., Carlsson, J., von Ballmoos, C., Högblom, M. 2018. Scavenging of superoxide by a membrane-bound superoxide oxidase. Nature Chemical Biology, 14(8):788–793.

Parasuraman, S., Thing, G., Dhanaraj, S. 2014. Polyherbal formulation: Concept of ayurveda. Pharmacognosy Reviews, 8(16):73–80.

Pereira, R. B., Taveira, M., Valentão, P., Sousa, C., Andrade, P. B. 2015. Fatty acids from edible sea hares: anti-inflammatory capacity in LPS-stimulated RAW 264.7 cells involves iNOS modulation. RSC Advances, 5:8981–8987.
Poongodi, T., Nazeema, T. H. 2019. Evaluation of free radical scavenging capacity and reducing power of polyherbal formulation comprising of three selected plants. *International Research Journal Of Pharmacy*, 10(4):143–149.

Que, X., Hung, M.-Y., Yeang, C., Gonen, A., Prohaska, T. A., Sun, X., Diehl, C., Määttä, A., Gaddis, D. E., Bowden, K., Pattison, J., MacDonald, J. G., Ylä-Herttuala, S., Mellon, P. L., Hedrick, C. C., Ley, K., Miller, Y. I., Glass, C. K., Peterson, K. L., Binder, C. J., Tsimikas, S., Witztum, J. L. 2018. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. *Nature*, 558(7709):301–306.

Troutman, T. D., Bazan, J. F., Pasare, C. 2012. Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell Cycle*, 11(19):3559–3567.

Venkatesan, T., Park, E. J., Choi, Y. W., Lee, J., Kim, Y. K. 2017. Anti-inflammatory activity of Ternstroemia gymnanthera stem bark extracts in bacterial lipopolysaccharide-stimulated RAW264.7 murine macrophage cells. *Pharmaceutical Biology*, 55(1):837–846.

Veres, B., Radnai, B., Gallyas, F., Varbiro, G., Berente, Z., Osz, E., Sumegi, B. 2004. Regulation of Kinase Cascades and Transcription Factors by a Poly(ADP-Ribose) Polymerase-1 Inhibitor, 4-Hydroxyquinazoline, in Lipopolysaccharide-Induced Inflammation in Mice. *Journal of Pharmacology and Experimental Therapeutics*, 310(1):247–255.

Yadav, U. C. S. 2015. Oxidative Stress-Induced Lipid Peroxidation: Role in Inflammation. In *Free Radicals in Human Health and Disease*, pages 119–129, New Delhi. Springer.