Research Article
ISSN (Print): 0971-6580
Toxicology International, *Vol 27(1&2), 14-22, 2020, DOI: 10.18311/ti/2020/v27i1&2/24457* ISSN (Online): 0976-5131

Therapeutic Role of Mitochondria Targeted Curcumin against *Pseudomonas aeruginosa* Induced Oxidative Stress in Thyroid Gland of Female *Swiss albino* Mice

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Abstract

Bacteria are found universally and contaminate the soil, air and water. Through contaminated air, water and soil bacteria reach inside the body and causes immense harm. The problem of thyroid is that, it affects the public health worldwide. The main purpose of this investigation is to find out the toxic effects of *Pseudomonas aeruginosa* on thyroid gland and preventive role of mitochondria targeted curcumin in bacterial induced thyroid toxicity in female *Swiss albino* mice. The animals are separated in different groups. Group I served as control and Group II administered with effective dose of *Pseudomonas aeruginosa.* Group III co-treated with mitochondria targeted curcumin for the period of 14 days. With the completion of treatment period blood serum and tissue samples were collected for determining the level of thyroid hormones and oxidative stress. In this investigation shows that mice exposed to bacteria have decreased T3 and T4 concentration as compared to control and co-treated mice have some elevated level of T3 and T4 hormones as compared to bacteria treated mice. In the biochemical study it is found that the increased lactate dehydrogenate, catalase activity, superoxide dismutase and lipid peroxidation and decreased glutathione reductase points towards the induced oxidative stress in thyroid gland. It is concluded that the exposure to *Pseudomonas aeruginosa* causes thyroid problem and treatment with mitochondria targeted curcumin prevents the induced toxicity.

Keywords: Mitochondria Targeted Curcumin, *Pseudomonas aeruginosa*, Thyroid Gland, T3, T4, TSH

1. Introduction

A butterfly shaped bilobed thyroid gland is found attached to trachea at anterior neck which regulates the basal metabolism of body via secreting the two hormones T3 and T4 in blood after stimulation by anterior pituitary with the help of chemical messenger TSH. The problem in thyroid gland is considered as the generic problem nowadays and affects the many people worldwide. The gut micro-biome diversity plays an important role for health and prevents from various diseases. The altered gut micro-biome results in disease. The microbial product short chain fatty acids are used as energy source to enterocytes, enterocytes along with thyroid hormone helps in their differentiation and enhance intercellular tight junction and in case of altered gut microbiota results in increased anti-inflammatory diseases[1].Bacteria are found everywhere in nature with great diversity. The different strains of bacteria are pathogenic and some of them are commensal. The pathogenic bacteria affect the humans and other plants and animals in different ways and affect their normal physiology. The titer of

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bacterial concentration in gut should be optimum at optimum temperature and pH. In case of any change in gut microbial titer the pathogenic bacteria attacks and disturbs the normal physiology. There are about 120 species of *Pseudomonas* which are pathogenic to humans are found in different habit and habitats with diversity[2]. The *Pseudomonas aeruginosa* is best suited to environment because of its ability to grow in poor nutrient conditions and extreme temperature. *Pseudomonas aeruginosa* is resistant to some antibiotics and nosocomial bacteria which affects the patients in many ways and generally grows in moistures.

The transmission of *Pseudomonas aeruginosa* is quite common about 30% of *Pseudomonas aeruginosa* was isolated from hydrotherapy pools, 72% from jacuzzis, spas and 38% from swimming pools in Northern Ireland, the other resources are whirlpools, wading pools and contact lens solutions and in hospitals the water supply is the important source of colonization and infection in susceptible patients[3-5].In the previous studies it has been showed that the bacterial cell and their metabolic products present in the extraneous environment of the host affect the thyroid functions due to secretion of anti-thyroid compounds[6]. Under normal condition the free radicals are minimized by cellular endogenous antioxidant defense system by enzymatic and non-enzymatic system. The enzymatic system includes SOD, CAT, GPx, GRx, GST and GSH and the non-enzymatic includes Vitamin-C and Vitamin-E[7]. The overproduction and accumulation of free radicals altered the cell viability by damaging the biological macromolecules which leads to ageing, degenerative diseases, thyroid dysfunction, inflammatory responses, brain dysfunction, cardiovascular disorders and cancer[8,9]. The normal curcumin binds to Thiamine Pyrophosphate (TPP) and can easily reaches to inner membrane of mitochondria. The capability of mitochondria targeted curcumin (mt-C) is greater than the curcumin. The present investigation has been undertaken to investigate the efficiency of mitochondria targeted curcumin over *Pseudomonas aeruginosa* induced thyroid gland toxicity in mice.

2. Material and Methods

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is aerobic Gram-negative and rod shaped bacteria. They do not ferment glucose and everywhere in the environment due to its ability to grow in poor nutrient condition and in high temperature also. These bacteria are nosocomial and affect the patients by skin infection. These bacteria are some antibiotic resistance and grow well in moisture.

2.2 Bacteria Culture

The bacteria *Pseudomonas aeruginosa* (MTCC 1748) are purchased from IMTECH Chandigarh and then cultured at the Endocrinology Lab, Department of Zoology, Dr. Harisingh Gour University, Sagar, M.P. The bacteria were cultured in antifungal agar medium after incubating them at 37°C for 18-24 hours. Then the broth culture streaked on nutrient agar and after 18-24 hours incubation the microbial colonies developed. The *Pseudomonas aeruginosa* produces smooth, large, translucent colonies with the greenish blue pigment over the nutrient agar.

2.3 Gram's Staining

A thin smear of the bacterial culture was prepared over a slide and fixed with heat. The smear was stained with crystal violet for 60 sec and followed by washing slowly with running tap water. Then Gram's Iodine added for 30 sec and then washed with tap water. After washing, slides were dipped in 90% ethanol for 10-15 sec and in the end; Saffranin was added for 30 sec and followed by washing with running tap water. After drying the slides were observed under the light microscope. Pink color indicated the presence of gram negative bacteria *Pseudomonas aeruginosa*.

2.4 Curcumin

Curcumin is a yellow colour component of turmeric, the spice which is derived from the roots (rhizomes) of plant *Curcuma longa*. Cultivation of turmeric in India is about 60% of the total area for spices and condiments. Traditionally turmeric is used in different disorder, due to its kaleidoscopic properties, such as a general tonic, stimulant and cosmetic and also used in the treatment of coughs, colds, asthma, arthritis, sore throats, wound healing and dyspepsia including peptic ulcers and as an antibiotic. Most of the infectious diseases are caused by pathogens and categorized as bacteria, virus, fungi, protozoa etc. in present day medicine system the drugs are as antiviral, antibiotics and antifungal and so on. Other than that the previous studies also reported that

the pathogens cause oxidative damage and curcumin is used as anti-infective agent[10]. The structure of curcumin is shown in Figure 1.

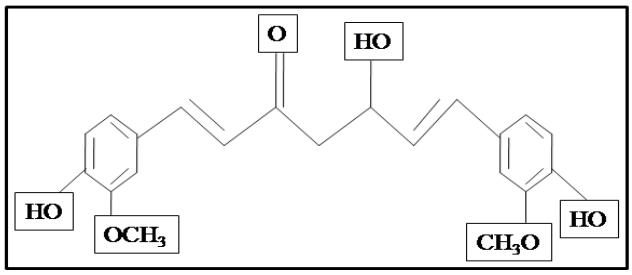


Figure 1. Structure of curcumin.

2.5 Mitochondria Targeted Curcumin

It is demonstrated and found that the most efficient way to reduce the oxidative damage is to use compounds with antioxidant properties[11]. Although the natural antioxidants such as; vitamin-E (tocopherol) and vitamin-C (Ascorbic acid) have less protective value. One of the potential explanations for this response, antioxidant therapies may be specific in nature and this is demonstrated[12]. After that it is found and reported that the antioxidants have limited potential because they do not accumulate sufficiently inside the mitochondria to reduce oxidative damage[13]. Thus, efficient mitochondria targeted antioxidants need to develop which have higher ability to reach the inner mitochondrial membrane. The structure of mitochondria targeted curcumin is shown in Figure 2.

Synthesis of mitochondrial targeted curcumin (mt-C) was performed by the method of Hercout (1988). Synthesis of mt-C, curcumin bind with the lipophilic cation TPP (Triphenylphosphonium Oxide). Curcumin (0.678 gm, 2 mMol) + TPP (0.524 gm, 2 mMol) Ph3P. HBr mixed in round bottom flask flushed with N2 sealed and heated at 70°C with magnetic string after 22 hrs dissolved in dichloromethane (10 ml), sample transferred to round bottom flask (25 ml), solvent evaporated in vacuum and give dark red oil (2.446 gm) mt-C, mt-G and Ph3PO along with lesser amount of TPMP and Ph3 present added Ethyl acetate (40 ml) heated at 70°C for 5-10 minutes solid material (1.7 gm) was dried under vacuum added methanol stirred with 30% aqueous H_2O_2 (7 ml) and added pyridine (0.13 ml) at room temperature for 21 hours. Added dichloromethane and $MgSO_4$ organic solvent was evaporated in vacuum and the mixture was dissolved, then extracted with aqueous HBr applied to a silica gel-G column evaporation with 5% rectified spirits in dichloromethane pure form of mitochondrial targeted curcumin (mt-C).

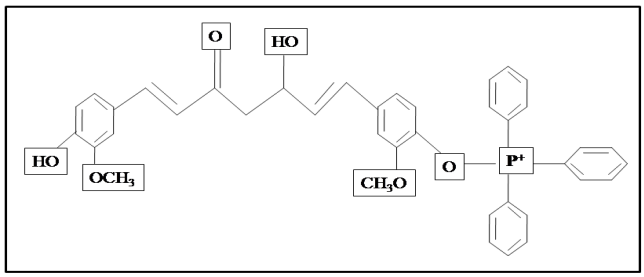


Figure 2. Structure of mitochondria targeted curcumin.

2.6 Experimental Animals

The animals female *Swiss albino* mice of average weight 28-32 gm were purchased from Veterinary College Mhow, Indore and housed in Department of Pharmaceutical sciences, Dr Harisingh Gour University, Sagar, MP, India with 12-12 hours dark-light cycle at 25 ± 2°C. Mice were fed with standard food and water. This work was approved by ethics approval committee with approval no. - AIPS/2015/2459/IAEC-03.

2.7 Experimental Design

The proposed experimental design for the present investigation is for 14 days and the mice are divided into three groups and each group has five female mice, the Group I served as control and treated with 0.02% DMSO, Group II served as DMSO + *Pseudomonas aeruginosa* (5 x 10^6/Kg bw/day, ip) treated and Group III served as DMSO + *Pseudomonas aeruginosa* (5 x 10^6/Kg bw/day, ip) + Mitochondria targeted curcumin (0.12 mg/kg bw/day, orally).

2.8 Body Weight

Body weight of mice were noted at an interval of two days starting from the day zero of treatment with the help of weighing balance and the individual values, as well as mean values were noted down. The values were expressed in gain weight/ animal.

2.9 Sample Preparation for Biochemical Assays

For biochemical studies, five mice from each group were sacrificed, thyroid gland dissected out and washes in ice cold saline solution (9%) and stored at -80°C. The thyroid gland extract was prepared in 0.02 M Tris-Cl (pH 7.4) and homogenate (10% w/v). Homogenate tissues were centrifuged at 1000 rpm for 10 min at 4°C and then, the collected supernatant re-centrifuged at 12500 rpm for 20 min at 4°C. The collected supernatant then stored in -80°C for the biochemical study.

Biochemical assays LDH, LPO, SOD and CAT. For LDH activity the adopted method[14] and then the optical density was measured at 340 nm for 10 min at room temperature. The LPO was performed as per the method of the absorbance taken at 548 nm[15]. The SOD activity was according to the methods and after that the 30 min incubation the OD was taken at 560 nm[16]. For catalase activity the OD was taken at 240 nm.

2.10 Serum for Hormonal Assays

The study of a thyroid hormone of blood sample from five mice in each group was collected by cardiac puncture into sterilized tubes and allows clotting at room temperature (25°C) then centrifuged at 1500 rpm for 15 min and serum collected for measurement of T3, T4 and TSH using ELISA kit provided by The Calbiotech Inc. (California, USA) as per the method applied[17]. All the assays were performed in triplicate.

2.11 Statistical Analysis

Results expressed as Mean ± SD and student t-test was applied for determining the level of significance between control and treated groups. Significance value expressed *p<0.05, **p<0.01, ***p<0.001.

3. Results

3.1 Bacteria Culture

The bacteria culture and gram negative culture plate of bacteria *Pseudomonas aeruginosa* is shown in Figure 3.

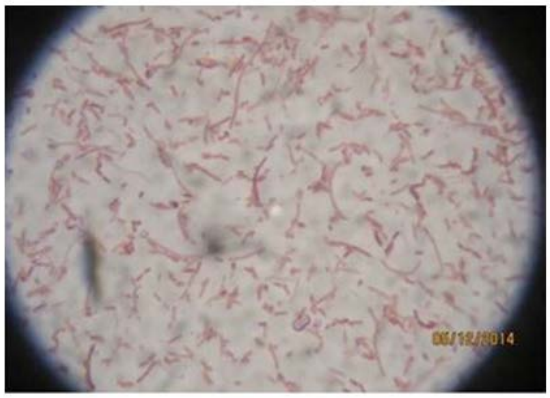


Figure 3. Gram negative culture plate of *Pseudomonas aeruginosa.*

3.2 Mitochondria Targeted Curcumin

The synthesis of mitochondria-targeted curcumin (mt-C); triphenylphosphonium cation covalently binds to alkyl chain of curcumin. So, these compounds are still hydrophobic in nature. The TLC result of curcumin binds to TPP is shown in Figure 4.

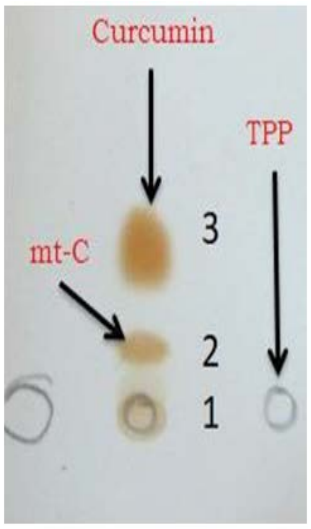


Figure 4. TLC of mt-C.

3.3 Net Body Weight Gain

After 14 days of treatment female mice treated with *Pseudomonas aeruginosa* (5 x 10^6/kg bw/day, ip) the body weight was increased. Co-treatment of mice with bacteria (5 x 10^6/kg bw/day, ip) and mt-C (1.63 mg/kg bw/day, orally) the adverse effect of bacterial toxicity reduced and decreased the net body weight.

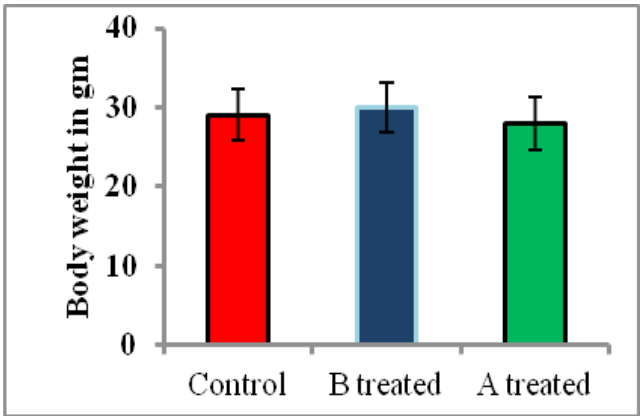


Figure 5. Effect of *Pseudomonas aeruginosa,* (Btreated) and antioxidant mitochondria targeted curcumin treatment (Atreated) on mice, weight increased and decreased respectively. The values represent mean + SD.

3.4 Serum Hormone Levels

A significant decreased levels of serum T3, T4 were observed after treatment with *Pseudomonas aeruginosa* to female mice ($p<0.01$) while the treatment with mt-C prevents the decreased level of T3 and T4 in serum significantly ($p<0.01$), However level of thyroid stimulating hormone does not change in treated group while treatment with mt-C increase the level of TSH in serum ($p<0.01$) significantly.

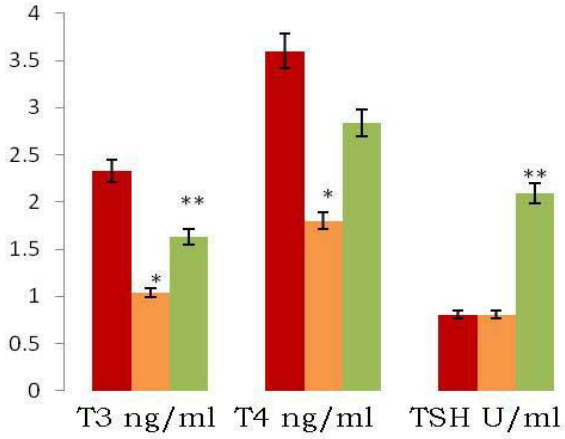


Figure 6. Effect of *Pseudomonas aeruginosa* (Btreated) and mitochondria targeted on mice serum, hormone levels in the group studied. Treatment of mitochondria targeted (Btreated), Level of T3, T4 ($p<0.01$) TSH significantly increase.

3.5 *Pseudomonas aeruginosa* Induced Altered LDH activity of Thyroid Gland by mt-C

LDH is a tetramer cytoplasmic enzyme, composed of H and M subunits. There are 5 different LDH isozymes which are selectively distributed namely LDH 1(H4) LDH 2(H3M), LDH 3(H2M2), LDH 4(HM3) and LDH 5(M4). The ratio of the subunit is tissue specific in well-defined. For spectrophotometric based analysis of LDH isozymes, the LDH activity obtained from extracts tissue of normal mice. *Pseudomonas aeruginosa* causes the significant increase the activity of LDH isozymes ($p<0.05$). However, the LDH activity significantly declines towards normal tissues of bacteria affected mice co-treated with mt-C. Treatment of normal mice with bacteria (5 x 10^6/kg bw/day, ip) for 14 days caused a significant increase LDH activity in thyroid gland as compared to control which indicates towards tissue injury. The level of LDH declined significantly in mice co-treated with bacteria and mt-C (1.63 mg/kg bw/day, orally) for 14 days

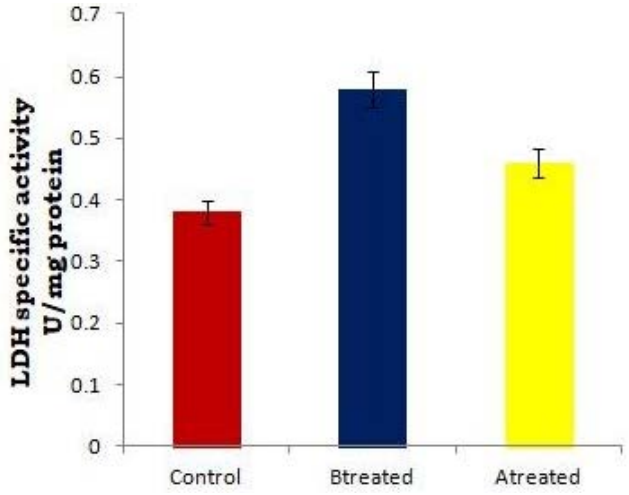


Figure 7. Effect of *Pseudomonas aeruginosa* (Btreated) and mitochondria targeted curcumin (Atreated) on the specific activity of lactate dehydrogenase in the thyroid gland. Data in panel represents mean± SD.

3.6 Amelioration of Bacteria Induced Oxidative Damage by Mitochondria Targeted

For determining the level of cellular oxidative damage and oxidative stress is measured as lipid peroxidation in terms of MDA is one of the widely accepted assays. Mice

treated with bacteria caused a significant increase the level of MDA ($p<0.001$) in thyroid gland compared to control group. Co-treatment with mitochondria targeted curcumin bacteria treated mice significantly decreased MDA level ($p<0.001$) in the thyroid gland.

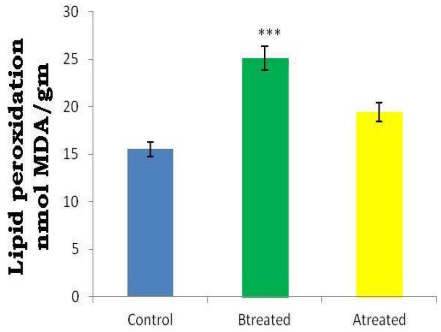


Figure 8. Effects of *P. Aeruginosa* (Btreated) and mt-C (Atreated), LPO in the thyroid gland. Data in panel represents mean± SD.

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) with multiple functions in living organisms. It is a carrier of an active thiol group in the form of a cysteine residue; it acts as an antioxidant either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS) and electrophiles or by operating as a cofactor various enzymes. Treated mice with bacteria caused a significant decrease the level of GSH in the thyroid gland whereas co-treatment of bacteria treated mice with mt-C prevent this decreased level of antioxidant GSH and maintained it towards normal level.

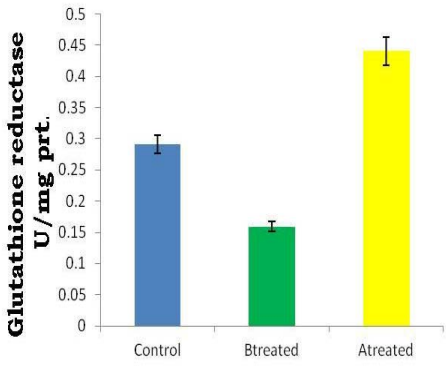


Figure 9. Effect of *Pseudomonas aeruginosa* (Btreated) and mitochondria targeted curcumin (Atreated) on the specific activity of glutathione reductase in the thyroid gland of mice. Data panel represents mean± SD.

The level of H_2O_2 assay is also used for determining the level of oxidative damage and oxidative stress at the cellular level. Treated mice with bacteria cause a significant increase H_2O_2 ($p<0.05$) in thyroid gland compared to control group. Co-treatment of mt-C with bacteria treated mice significantly prevented the increase H_2O_2 in the thyroid gland and the measured activity of catalase.

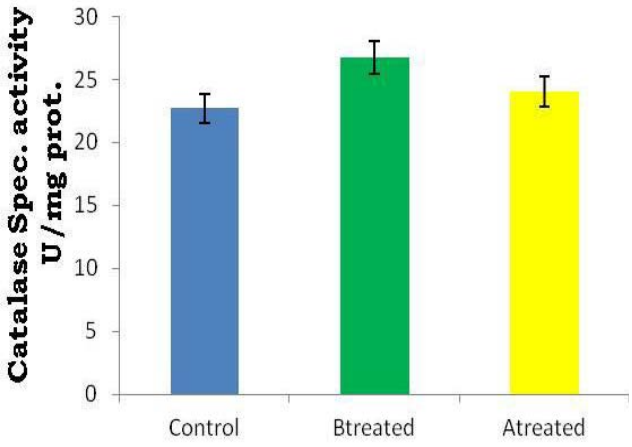


Figure 10. Effect of *Pseudomonas aeruginosa* (Btreated) and mitochondria targeted curcumin (Atreated)on the specific activity of catalase in the thyroid gland. Data in panel represents mean± SD.

3.7 Mitochondria Targeted Curcumin Treatment Ameliorates the Bacteria Induced Level and Expression of SOD in Thyroid Gland of Mice

Superoxide dismutase is an enzyme which catalyzes the superoxide (O^{2-}) into oxygen and hydrogen peroxide. Oxidative stress and mitochondrial dysfunction are known to produce superoxide anions. SOD is the first enzyme which neutralizes the superoxide anion free radical (O^{2-}) generated during the cellular oxidative stress. According to the Figure the level of active SOD significantly increases ($p<0.01$) in the thyroid gland. However, co-treatment of bacteria treated mice with mt-C significantly prevents this increased level of total SOD in the thyroid gland.

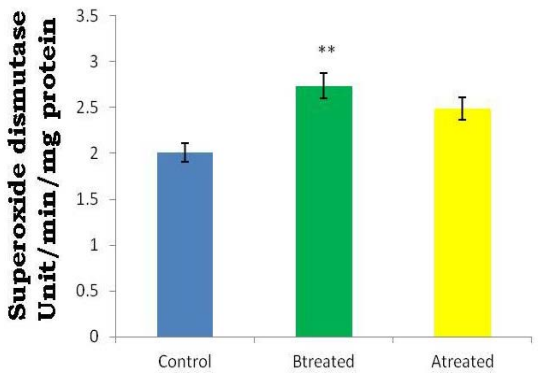


Figure 11. Effect of *Pseudomonas aeruginosa* (Btreated) and mitochondria targeted curcumin (Atreated) on the specific activity of superoxide dismutase on the thyroid gland of mice. Data panel represents mean± SD.

4. Conclusion and Discussion

The previous studies has been demonstrated that the mitochondrial targeted antioxidants like mitochondria targeted curcumin shows better results as compare to the non-targeted antioxidants due to their easy route to inner mitochondrial membrane[18,19]. The present investigation of 14 days treatment to mice with *Pseudomonas aeruginosa* shows decreased serum T3 and T4 hormone concentration and increased body weight which means bacteria inhibits the DNA replication which altered the concentration of enzyme, structural proteins and receptors. All these are due to the reduced food and water intake and decreased protein levels and altered metabolic activities. Previously it is also reported that the mt-C inhibits the increased body weight after treatment with bacteria[20,21]. The previous studies has been reported that the intake of contaminated food and water affects growth and development of individual and the thyroid hormones T3, T4 and TSH are the parameters of growth and development of the individual[22,23]. The results of the present studies shows that the serum concentration of T3 and T4 decreased and no change has been observed in TSH level which means the bacterial toxicity affects the growth and development and causes hypothyroidism. The thyroid follicles contains poly unsaturated acid and has high oxygen supply so lipid peroxidation is rapid and reduced T3 and T4 is indicator of induced oxidative stress in follicles but no changes hypothalamo-pituitary-thyroid axis because *Pseudomonas aeruginosa* secretes enterotoxin which binds to kinase receptor has no effect on cAMP hence the hypothalamus-thyroid-pituitary axis is not affected and the level of TSH is constant. In the previous study it has been shown that during the normal cell proliferation and pathological condition the uptake of glucose and process of lactate formation increase many folds the lactate dehydration helps in converting pyruvic acid into lactic acid and lactic acid into pyruvic acid[24]. The lipid peroxides are considered to be the markers of oxidative damage and curcumin interacts with induced free radicals and neutralize them by inhibiting the lipid peroxidation[25,26]. The result of the present investigation shows a linear view between LDH and MDA concentration in thyroid gland extracts of mice exposed to bacteria *Pseudomonas aeruginosa* indicates that the bacteria induces toxicity in thyroid gland of mice increasing the lipid peroxidation and this is also reported in previous research works[27]. In the present investigation the results shows the increased lipid peroxidation plays an important role in modifying the LDH activity and increased lactate production due to toxin secretes by bacteria induces cellular oxidative stress. The results of the present investigation showed that the mitochondria targeted curcumin inhibits the lactate production. The cellular oxidative stress is generally associated with the over production of ROS and reduced accomplishment of antioxidant defense system. In the previous research it is also reported that the concentration of thyroid hormone is regulated by oxidant and anti-oxidants and protect the cell[28]. LPO is an oxidative stress biomarker and previously it is reported that mice treated with bacteria have significantly elevated MDA level[29]. In the present investigation *Pseudomonas aeruginosa* treated mice have increased MDA level and mice co-treated with mitochondria targeted curcumin have reduced MDA level as compared to the bacteria treated mice. The level of GSH is decreased in bacteria treated mice due to reduced glutathione loss electrons by induced oxidative stress and the results of present investigation showed the mice co-treated with mitochondria targeted curcumin increased the GSH level and protect the mice from bacteria induced thyroid toxicity in mice. The previous research has been showed that the excessive production of free radicals reacts with bio macro molecules and damages those[30]. The level of SOD and CAT increases in case of bacteria *Pseudomonas aeruginosa* induces hypothyroidism in mice and mitochondria targeted curcumin neutralizes the thyroid gland toxicity. The

results of this investigation and previously published work it is concluded that the bacteria *Pseudomonas aeruginosa* induce toxicity in thyroid gland of mice and bacteria treated mice co-treated with mitochondria targeted curcumin prevents from toxicity. This investigation is found that the mitochondria targeted curcumin shows preventive effects against bacteria generated thyroid problem in mice. In the previous study it is found that the mitochondria targeted curcumin also helps in reducing the oxidative damage in mitochondria of thyroid gland of mice against *Escherichia coli* induced toxicity[31]. Previous work and present works are concluded that mitochondria targeted curcumin plays an effective prevention against bacteria induced toxicity in thyroid gland.

5. Acknowledgement

This study was performed in the Department of Zoology, Dr Harisingh Gour University Sagar (M.P.). In this study the fourth and corresponding author helps in design the experiment, the contribution of first author helps in writing the manuscript and statically analysis, the second author perform the experiment, third author helps in bacterial culture and final rearranging the manuscript. This study was sponsored and supported by UGC Non-NET Fellowship with the help and support by Department of Zoology.

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