Response of the antioxidant system in *Oreochromis mossambicus* reared on gold nanoparticle fortified diet.

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ABSTRACT

Nanotechnology involves the application of materials at the nanoscale to new products or processes. The interest in nanomaterials is a result of the extreme dependence of properties (electronic, magnetic, optical, mechanical, etc.) on particle size and shape in the 1-100 nm regime. The 1-100 nm scale is of interest for biological interfaces because objects less than 12 nm in diameter may cross the blood-brain barrier [.Sonavane ,et al;2008] and objects of 30nm or less can be endocytosed by cells [Connor,et al; 2005].However the impact of these nanomaterials on environmental health remains unclear.(Maynard,et al;2006).An increasing number of scientific reports have appeared in the last decade that highlight this issue, with the goal of understanding the interactions between different types of nanoparticle and cells as function of size, shape, and surface chemistry of the nanomaterial[Lewinski,et al;2008]. The environmental risk assessment of these new materials involves the identification of associated hazards as well as the routes leading to exposure. Currently large gaps exist in our knowledge and understanding of the toxicity and exposure of nanomaterials for aquatic organisms, which hinder their risk assessment. Presently ecotoxicological studies with gold nanoparticles are rather limited with only a few reports of aquatic organisms. Hence, in the present study, an attempt was made to assess whether there is any toxic effects on synthetic and biologically synthesized gold nanoparticle supplementation on fish Oreochromis mossambicus.Gold nanoparticle were prepared using 0.3 M HAucl$_4$3H20 (Sigma-Aldrich). Synthetic gold nanoparticles were prepared by soduim borohydride reduction method and for the green synthesis ,aquous extract of Ocimum sanctum and Curcuma longa were used. Synthesis of gold nanoparticles were confirmed from the UV-Vis study of surface plasmon resonance property of the colloidal solution. Juveniles of Oreochromis mossambicus in the range 7+- 0.35cm and 5+-0.62gm were collected from ADAK, Varkala, quarantined and stocked at 20 fish/1000L tanks and maintained at laboratory conditions. Experimental diet was prepared by incorporating 10ml of biogenic and synthetic gold nano solution per 100gms of basal feed. Non-treated control diets and biogenic gold nanoparticle incorporated diets too were prepared. The experimental schedule was for six weeks and the fishes were fed at 2% of body weight twice daily. The biological effect was assessed in terms of selected antioxidant indices .The biological effect was assessed in terms of expression levels of antioxidant enzymes Catalase, Reduced glutathione, LDH and Lipid peroxidation.

Index terms*: Emblica officianalis , Gold nanoparticle Green synthesis, Nanotechnology.*

1. INTRODUCTION

The recent development and implementation of new technologies have led to new era, the nano-revolution which unfolds role of plants in bio and green synthesis of nanoparticles which seem to have drawn quite an unequivocal attention with a view of

synthesizing stable nanoparticles (Kavitha, *et al;*).The possibilities of employing plants in the deliberate synthesis of nanoparticles have burgeoning interest as an important source towards reliable and environmentally benign method of metallic nanoparticles synthesis and its characterization. Nanoparticles have diverse applications in life sciences such as drug development, protein detection and gene delivery. Drug targeting through nanoparticles may improve therapies yet a thorough understanding of the feature that regulates the effect of carrier nanoparticle is needed to translate this approach into the clinical application. Currently large gaps exist in our knowledge and understanding of the toxicity and exposure of nanomaterials for aquatic organisms, which hinder their risk assessment. Therefore, increased research is warranted on various toxicological issues related to nanoparticles. Nanofeed applications could be used to improve the delivery of micronutrients or unstable ingredients. In the present study, the plant selected for biogenesis of gold solution is *Curcuma longa.* and *Ocimum sanctum.* .The objective of the study is to determine the effects of biogenic gold nanoparticle in the aquatic environment on *Oreochromis mossambicus* with emphasis on selected antioxidant indices. The biological effect was assessed in terms of expression levels of antioxidant enzymes Catalase(CAT),Reduced glutathione(GSH), Lactate dehydrogenase(LDH) and Lipid peroxidation.(LPO).

2. MATERIALS AND METHODS

2.1 Synthesis of gold nanoparticles

Synthetic gold nanoparticles are prepared by the reduction of Auric chloride (HAuCl4]). After addition of reducing agent(here sodium borohydride),the solution is rapidly stirred and it leads to the reduction of gold ion ($Au^{3+)}$ to neutral gold atom(Au^0) and the continuation of the operation will turn out all the gold ions to neutral atoms and the solution becomes supersaturated. An addition of sodium borohydride saw an immediate change in the solution colour from yellow to purple (Fig: 1, 2, and 3). Tri sodium citrate is used as capping agent. The formation of colloidal gold nanoparticles was investigated using UV -Vis absorption. In the present study, green process for the production of gold nanoparticles uses direct interaction of $HAuCl_4$ with aquous extract of *Curcuma longa* and *Ocimum sanctum* in the absence of manmade chemical and thus satisfies all the principles of 100% green chemical process. Various phytochemicals present in the plant extracts presumably responsible for making a robust coating on gold nanoparticles and thus rendering stability against agglomeration. Absorption measure indicated that plasmon resonance wavelength of synthetic gold nanoparticles and green synthesized gold nanosolutions from both the plant extracts is 520nm.Experimental diets were prepared by incorporating10 ml of the above mentioned concentrations of gold nanosolution per 100gms of basal feed prior to pressure pellatisation.

(Fig:1)

(Fig:2)

(Fig:3)

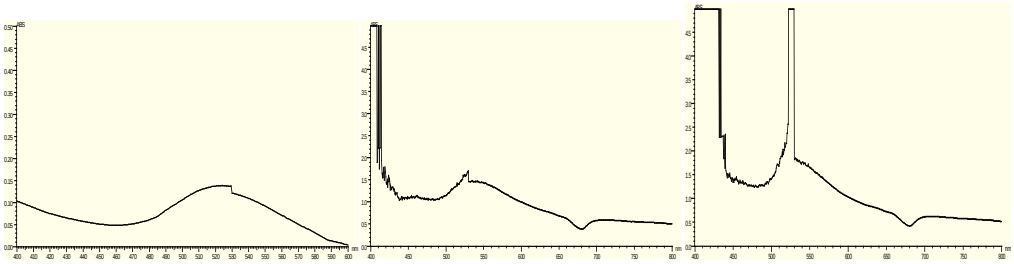
(**Fig:4** Synthetic gold nanoparticle, **Fig:5**.Biogenic gold prepared from *Curcuma longa* , **Fig:6..**Biogenic gold from *Ocimum Sancta*)

2.2 UV-Vis Spectrophotometric analysis of Gold Nanoparticle:

Spectrophotometry is an important aspect of characterization of gold nanoparticles. With increase in particle size, the absorption peak shift to longer wavelength and the width of the absorption spectra is related to the size distribution range. Generally gold nanoparticles display a single absorption peak in the visible range between 510-550 nm because of the surface plasmon resonance and show heavy absorption of visible light at 520 nm. This gives purple colour .

The appearance of violet colour is the evidence of the formation of gold nanoparticle in the reaction mixture and the efficient reduction of Au^{3+} to Au^0 (Fig: 1, 2 and 3)). The formed colour solution allowed to measure absorbance against wavelength to confirm the formation of gold nanoparticle. The corresponding UV absorption spectra of the nano gold solutions in experiment is shown in (Fig: 4, 5 and 6). The absorption is a typical gold surface plasmon vibration excitation for colloidal gold nanoparticles when they interact with electromagnetic radiation. In the optical absorption spectrum of the resultant nanoparticles the absorption wavelength of gold nanoparticles were observed at 520 nm.. The absorption spectrum of both the green synthesized nanoparticles is shown in fig 5 and 6.In both the reaction mixtures the observed intensity of SPR peak is more with small sharpness in the peak suggesting complete

reduction of gold nanoparticles. The reasonable narrow absorption peak indicated that the particles were not aggregated and the capping was effective.. Phytochemical constituents in the plants and spices extract like essential oils (terpenes eugenols etc), polyphenols and carbohydrate contain active functional groups, such as hydroxyl aldehyde and carboxyl units play important role for reduction of HAucl4 to gold nanoparticles. Gold nanoparticles particles produced using phytochemicals or other extract components remain stable for prolonged period.



(**Fig:4)** **(Fig:5)** **(fig:6)**

(Fig 4. UV-VIS Spectrum of Chemically reduced GNP, **Fig 5.**UV -VIS Spectrum of nano *Ocimun sanctum* **, Fig:6**.UV -VIS Spectrum of nanocurcuma)

2.3.Experimental design

Orechromis mossambicus juveniles belonging to the brood stock were obtained from the ADAK centre,Varkkala,TVPM,Kerala**.**Sixty fishes belonging to both sexes and having an initial length of about 8 - 9 cm and 6-7 gm weight were selected. The experimental setup consists of six tanks with 10 fishes per 10 litre of water. Basal feed was prepared as out lined by Hardy et al. (1978). The fish in each set was fed with 2% body weight per day. First tank served as control .In the second and third, tanks fishes were fed with diet containing gold nano solution prepared using *Curcuma longa* and *Ocimum sanctum* extract and the fourth and fifth tanks were fed with aquous extract of *Curcuma longa* and *Ocimum sanctum* containing diets.Sixth tank was maintained on chemically reduced gold nanoparticle incorporated diet. After 30 days of treatment,major biochemical changes with respect to selected enzymes were estimated.

2.1.Parametres investigated:

1.Assay of Catalase

Catalase level in different tissues were determined using the method of Maehly and Chance 1955. The estimation was done by spectrophotometry following the decrease in absorbance at 230 nm. Specific activity was expressed as International units/mg protein.

2.Assay of Reduced glutathione

Reduced glutathione was estimated by the method of Beutler *et al.* (1963). Absorbance of reactants was read at 412 nm against blank.. Values were expressed as µg of reduced glutathione µg protein-1.

3.Assay of Lactate Dyhydrogenase(LDH)

Lactate Dyhydrogenase(LDH) was assayed according to the method of King(1965). The enzyme activity was expressed as µ moles of pyruvate liberated / hr / mg protein

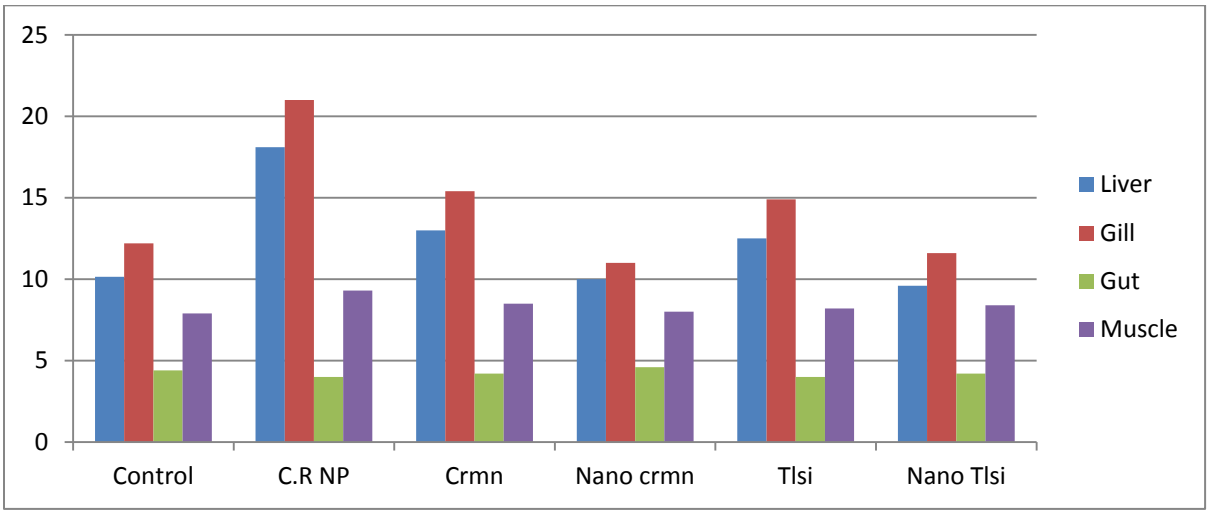
4.Assay of lipid Peroxidation(LPO)

Lipid peroxidation(LPO) was determined by the method of Utley,*et al*;(1967). Absorbance of the reactants was read at 535 using a reagent blank. Values were expressed as nmoles of thiobarbituric acid reactive substances (TBARS) formed hour-1mg protein-1.

3.RESULTS

3.1.Catalase(CAT)

Fig:1 Effect of biogenic gold nanoparicles on CAT activity in selected tissues of *Oreochromis mossambicus*



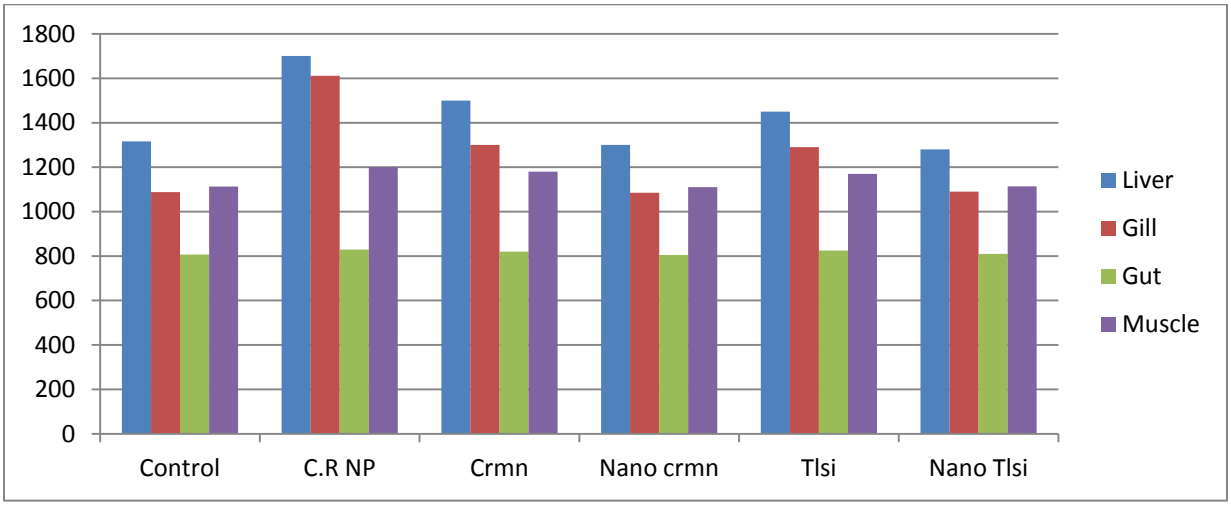
(CRNP:Chemically Reduced Nanoparticle, Crmn:Curcumin(*Curcuma longa*)Nano Crmn:Biosynthesized Nanocurcumin.,Tlsi :Tulsi(*Ocimum sanctum*),Nano tlsi:Nano Tulsi)

Each value represents mean ±SD of 3 separate experiments.Values were represent as u/mg of protein

In the present study catalase activity in different tissue of *Oreochromis mossambicus* treated with chemically synthesized and green synthesized (using aqueous solution of *Curcuma longa* rhizome extract and *Ocimum Sanctum* leaf extract)showed marked variations (Fig:1 ,Table: 1) compared to control groups.CAT activity was significantly high ($p<0.05$) in gills of *O.mossambicus* treated with chemically synthesized gold nanoparticle encapsulated food fed groups compared to control groups and groups treated with different plant extracts. Comparison between the nanocurmin and nano tulsi fed groups ,there was no significant difference in CAT activity and the measures comes closer to that of control groups.Response of biogenic nanotreated groups suggests that they are more potent in CAT activity.

3.2.Reduced glutathione(GSH)

Fig:2 Effect of biogenic gold nanoparticles on Reduced Glutathione(GSH) content in *Oreochromis mossambicus*



(CRNP:Chemically Reduced Nanoparticle, Crmn:Curcumin(*Curcuma longa*)

,Nano Crmn:Biosynthesized Nanocurcumin.,Tlsi :Tulsi(*Ocimum sanctum*),Nano tlsi:Nano Tulsi)

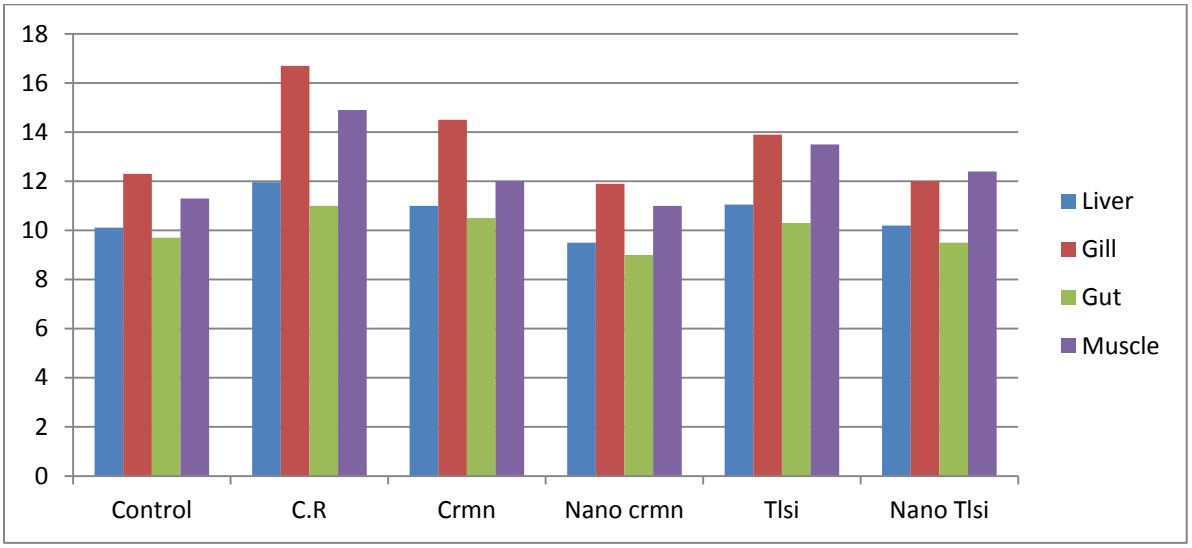
Each value represents mean ±SD of 3 separate experiments.Values were represent as u/mg protein

Figure 2 and Table 2 shows the effect of synthetic ,biogenic gold AuNPs and aquous extract of *Ocimum sanctum* and *Curcuma longa* on reduced glutathione concentration in various tissues in *O.mossambicus*

The chemically synthesized AuNPs caused a statistically sisignificant increase in reduced glutathione ($P<0.05$) on tissues under study when compared to the effects of green synthesised gold nanoparticles .The increase in reduced glutathione was the highest in liver followed by muscles.In the present study, synthetic gold nanoparticle treatment caused a significant increase in reduced glutathione which may be due to increase in lipid peroxidation in the liver .Increased cellular GSH levels can confer cells resistance and decreased cellular GSH levels can sensitize cells to the killing effects (Chiba,*et al.*, 1996; Yang,*et al*., 2000).

3.3 . LACTATE DEHYDROGENASE (LDH).

Fig:.3 .Effect of biogenic nanoparticles on LDH activity in *Oreochromis mossambicus.*



(CRNP:Chemically Reduced Nanoparticle, Crmn:Curcumin(*Curcuma longa*)

,Nano Crmn:Biosynthesized Nanocurcumin.,Tlsi :Tulsi(*Ocimum sanctum*),Nano tlsi:Nano Tulsi)

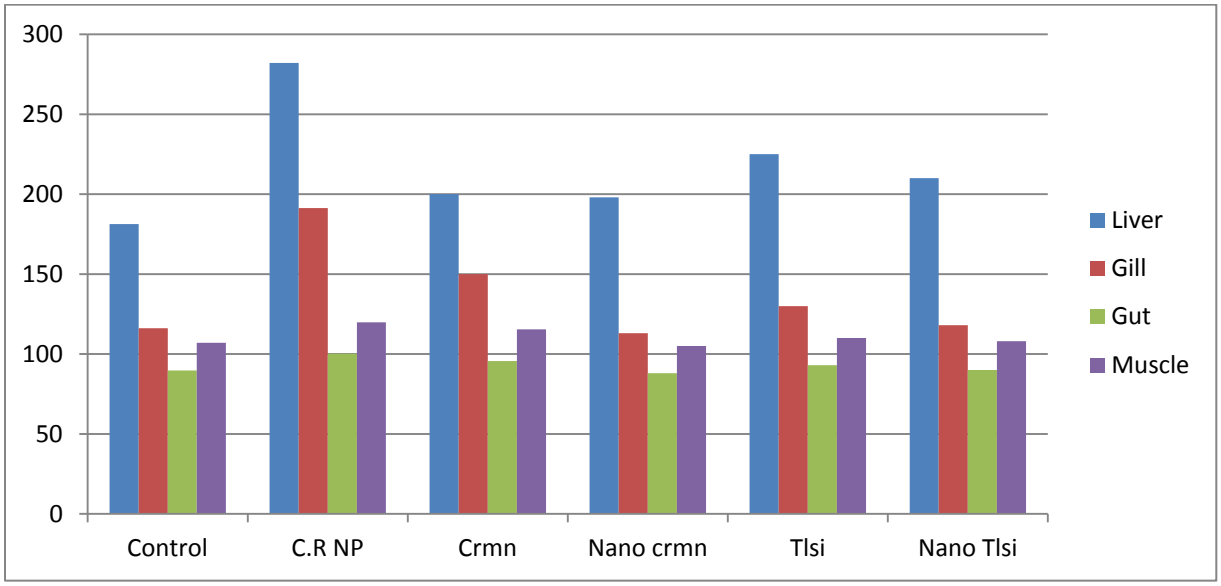
Each value represents mean ±SD of 3 separate experiments.Values were represent as u/mg of protein.

There are changes in the LDH level among the treated groups and control.Significantly high ($P<0.05$) LDH level is found in all the tissue of groups treated with synthetic nanoparticle fed groups.Among the tissues, gill and muscle showed significantly elevated LDH level.Comparison between the groups treated with nanocurcumin and nanotulsi,nanocurcumin treated group shows bettere LDH profile.LDH measures of aquous extract of *Curcuma longa* and *Ocimum sanctum* are closer to the control.

3.4 LIPID PEROXIDATION(LPO)

Fig:4 Effect of biogenic nanoparticles extracts on Lipid peroxidation (LPO)in

Oreochromis mossambicus



Each value represents mean ±SD of 3 separate experiments

Values were represent as n mole/mg protein

In the study LPO values are found to be significantly high ($p<0.05$) in all the tissues especially in liver in the groups supplemented with synthetic nanoparticle incorporated food. The data suggests that expression of Lipid peroxidation is nill in nanocurcumin and nanotulsi fed groups. The level of lipid peroxidation in *Curcuma longa* and *Ocimum sanctum* groups seems to be those values of control.

4. DISCUSSION

The application of nanotechnology in biology encompasses development of nanomaterials for delivering and monitoring biologically active molecules, disease staging, therapeutically planning, surgical guidance, neuro-electronic interfaces, and electronic biosensors (Huang *et al.*,2010). However, it is also essential to understand the advantage of green synthesized gold nanoparticles over chemically synthesized nanoparticles on the biological system. Oxidative stress is a causative factor for many diseases and underlying pathologies. In this study, chemically synthesized gold nanoparticles were found to induce significant oxidative stress in the selected tissues of fishes. Oxidative stress plays important roles in cellular signaling, inflammatory, and genotoxic and proliferative responses (Knaapen,*et al.*,2004,Zhong,*et al.*,1997).Nanoparticles have the potential to interact with the biological system and cause undesirable effects. One of these damaging effects could be the disturbance in the natural balance between oxidative stress and antioxidant defense indices which in turn can lead to various pathologies.

To cope with elevated oxidative stress, cells mount protective or injurious responses. For instance, cells activate enzymatic and non-enzymatic antioxidant defense mechanisms like glutathione peroxidases, catalases, etc (Huang,*et al.*, 2010). Oxidative stress has been identified as a likely mechanism of nanoparticle toxicity (Li,*et al.*, 2008).

Figure :1 represent the changes in the action of CAT in different tissues of *O.mossambicus* .Catalase is the enzyme responsible for dissipation of hydrogen peroxide formed during oxidative stress The results show that there is elevated CAT activity in synthetic gold nanoparticle fed groups compared to control which suggests occurrence of oxidative stress.CAT activity was elevated for the detoxification of increased H_2O_2.CAT is the most abundant peroxisomal enzyme. since peroxisomes play key role in many cell functions, it may have multiplied in stress. It may be the one of the reason of increase in CAT activity. Increase of CAT is reported in

some fish species under oxidative stresss (Gull,*et al*;2004,Zhang,*et al;*2004).The response patterns of groups treated with aquous extract of *Curcuma longa* and *Ocimum sanctum* are very closer to the control .The data reveals gill showed highest antioxidant activity in the groups treated by synthetic gold nanoparticles. This suggests the adaptive response to protect the fish from free radical toxicity induced by synthetic gold nanoparticles. In contrast to these results, biogenic nanoparticles prepared from aqueous extract of *Ocimum sanctum* and *Curcuma longa* caused no significant changes in the catalase activity in all the tissues studied. Since catalase was not significantly altered by biogenic nanoparticle treatment. It reveals that H_2O_2 might not have been generated in significant amount by the biogenic AuNPs to elicit an alteration in the catalase activity. Therefore, the biogenic gold nanoparticle in this study may not be toxic to all the organs in fishes. These results are in agreement with the study of Shukla,*et al;*(2005) indicating their potential for application in nanoimmunology, nanomedicine, and nanobiotechnology.

Increased cellular GSH levels can confer cells resistance and decrease cellular GSH levels can sensitize cells to killing effects.(Chilba,*et al;*1996,Yang,*et al ;*2000.).GSH is a major cytosolic low molecular weight sulphydryl compound that acts as a cellular reducing and a protective reagent against numerous toxic substances. Liver and muscle show elevated GSH level when treated with synthetic gold nanoparticles compared to control.(Fig 2.)As a result of scavenging the ROS,GSH is oxidized and form glutathione protein disulphide. Since there is high consumption of GSH, to manage the oxidative stress, cells try to reduce or synthesize more GSH. Thus reduced GSH content is a biological index to indicate exposure to contaminants. Plots of biogenic nanoparticles from *Ocimum sanctum* and *Curcuma longa* extracts suggests that they produce no oxidative stress and they are more potent than their non nano counter parts. Since GSH resist the reactive oxygen toxicity .The different level of Total Glutathione can serve as marker of exposure to the substances which disturb piscine oxyradicals.

LDH enzyme patterns are portrayed in Fig :3.In the study elevated levels of LDH in synthetic nanoparticle treated groups reveals the increased permeability of the cells and cellular leakage .An increase in level of LDH reflects damage to tissues and oxidative stress. It was observed that biogenic gold nanoparicles from both the plant extracts (*Curcuma longa* and *Ocimum sanctum*) have reversal effects on the levels of LDH.The tissues of groups treated with *Ocimum sanctum* and *Curma longa* also exibit a better LDH profile.

LPO has been shown to cause various negative effects in terms of cellular integrity in the membranes of the cells as well as other changes such as the product of pro inflammatory agents and toxic substances.(Greenberg,*et al ;*2008).LPO is commonly used as an indicator of oxidative stess in cells and tissues.(Botsglou *et al;*1991).The level of LPO is compared among the different treatment groups of *Oreochromis mossambicus* in Fig:4.The study reports indicates that fish exposed to synthetic AuNPs induce a greater risk of oxidative stress with increased levels of Lipid peroxides.The LPO level is found high in liver. Since liver is an important organ for storage of iron, it may be susceptible to lipid peroxidation than other tissues. Gold nanoparticles are taken up by the Kupffer cells of the liver and their bioaccumulation is regulated by the reticuloendothelial system.The LPO levels did not show much difference between control and biogenic gold nanoparticle fed groups.The group treated with plant extracts alone also shows results that don't vary much from control.

All the parameters showed increase when treated with synthetic nanoparticles compared to the control groups and groups treated with green synthesized nanoparticles. In this study, liver and gill was the organ most sensitive to the effects of synthetic gold nanoparticles. Liver of synthetic gold nanoparticles treated fish showed a significant increase in lipid peroxidation and reduced glutathione and gills of the same group show increase in CAT and LDH activity when compared with the fishes of other groups. These results points to the fact that synthetic nanoparticles induce tissue damage. No evidence indicates that biogenic gold nanoparticles cause toxicity at the cellular level. The response patterns suggests biogenic nanoparticles from *Ocimum sanctum* and *Curcuma longa* are more potent than normal *Ocimum sanctum* and *Curcuma longa* aquous extracts as an anti oxidant and also reveals nano form of the plant extracts increases the bio availability of the constituents present in them. The versatile phytochemical mediated green nanotechnological process has been shown to be effective in both the generation and stabilization of non-toxic gold nanoparticles for direct applications in a myriad of diagnostic and therapeutic applications. The study reveals the interaction of synthetic Nanoparticles with biosystems plays an important role in triggering toxicity through a range of mechanisms, including membrane perturbation and resultant oxidative stress.

The better enzyme profile of groups treated with biogenic nanoparticles can be attributed to the bio transformed gold nanoparticle present in it. The plots of groups treated with synthetic nanoparticles clearly indicates the toxic effect of chemical used for AuNP synthesis. The plant mediated stabilized and capped gold nanoparticles may cross the barrier of cytotoxicity This highlights the importance and advantage of green synthesis of nanoparticles over chemical reduction. Biogenic nanostructures have been considered to have better biocompatibility than the chemically synthesized nanostructures.

5.CONCLUSION

Gold nanoparticles have a wide range of applications in various fields. It is therefore essential to study their interaction with the biological system. In the present study, the effect of gold nanoparticles was studied on oxidative stress and antioxidants in various tissues of fish.

The synthetic gold nanoparticles used in this study caused a significant change in the oxidative stress and antioxidant defense indices in all the tissues examined. From the results it can be concluded that the activities and expression levels of antioxidant enzymes and oxidative stress can be used as biomarkers to evaluate the influence of nanoparticles on the biochemical pathway and enzymatic function in *Oreochromis mossambicus* .The activity of antioxidant may be increased or inhibited under chemical stress depending on the intensity and duration of stress applied. Thus, it is hoped that, further research in the fish model developed will strengthen and expand the knowledge on biogenic gold nanoparticle. Studies in this direction could help to shape the future of aquaculture. Green synthesized gold nanoparticles showed less toxicity compared to other groups .Thus it shows that biogenic gold nanoparticles can be looked up as an environmentally benign replacement to the toxic chemical methods for the synthesis of nanostructures and as promised candidates for biomedical applications. Our results have demonstrated the property of phytochemicals, present in *Ocimum sanctum* and *Curma longa* to reduce the gold metal, to the corresponding gold nanoparticles. The versatile phytochemical mediated green Nano technological process has been shown to be effective in both the generation and stabilization of non-toxic gold nanoparticles for direct applications in a myriad of diagnostic and therapeutic applications

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