PHYTOTOXICITY IN RESPONSE TO NANOPARTICLES IN RICE AND THEIR IMPACTS ON ANIMAL MODEL

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By

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CERTIFICATE

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I dedicate this thesis to my beloved mother who is the reason behind my every achievement and my respected and loveable father who stood beside me at every moment in my life


بسم الله الرحمن الرحيم

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Rubab Zahra

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LIST OF ABBREVIATIONS

Ag	Silver
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Transferase
AST	Aspartate Amino transferase
BUN	Blood Urea Nitrogen
Ce	Cerium
CHOL	Cholesterol
CREA	Creatinine
Cu	Copper
EDX	Energy Dispersive X-ray Spectroscopy
ENPs	Engineered Nanoparticles
Glu	Glutamine
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HNO_3	Nitric Acid
ICP	Inductively Coupled Plasma−Optical Emission Spectroscopy
mRNA	Messenger Ribonucleic Acid
NP	Nanoparticles
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase Activity
TBILI	Total Bilirubin
TNPs	Titanium Dioxide nanoparticles
TP	Total Protein
XRD	X-ray Diffraction
ZnO	Zinc Oxide

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ABSTRACT

Different applications of nanotechnology has led to various environmental concerns despite its some positive effects in agriculture and poultry to improve growth and development of plants and chicken. The aim of present work was to assess the phytotoxicity in response to TiO_2 nanoparticles (TNPs) application and effects on chicken upon exposure to TNPs through feed highlighting food chain contamination. The parameters considered were root and shoot length, plant biomass, chlorophyll content, uptake in grains and changes in root morphology. Impacts on local chicken were assessed by their growth performance and biochemical blood profile. TNPs were synthesized through liquid impregnation method and characterization was done using X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX). Rice was grown in soil amended with TNPs (particle size 17-30 nm) to develop concentration levels of 0, 500, 600, 700, 800, 900 and 1000 mg kg^{-1}. Chicken were given dosages of 0, 10, 20, 40 and 80 mg kg^{-1} of chick body weight for 45 days. The growth of rice was enhanced up to 700 mg kg^{-1} and decreased at higher concentrations. ICP results detected no Ti in grains while SEM images confirmed that TNPs enhanced root hair development. TNPs showed no adverse effect on chicken development as well as biochemical parameters, there was no significant difference between control and treated groups except for Blood Urea Nitrogen (BUN). Significant decrease in BUN levels can be attributed to disturbance in kidney or liver functioning induced by TNPs.

Chapter 1

INTRODUCTION

1.1 Background

Nanotechnology is manipulation of matter at its atomic scale for innovative characteristics and utilization in medicine, agriculture, industries and manufacturing sector. The basic principle of the nanotechnology involves reducing the particle sizes, while improving efficiencies of cellular uptake and other physical properties (Iavicoli *et al.,* 2012; Vasantharaja *et al.,* 2014). Nanotechnology is useful in providing technological platforms for research and revolution of biological systems (Jalill and Yousef, 2015).

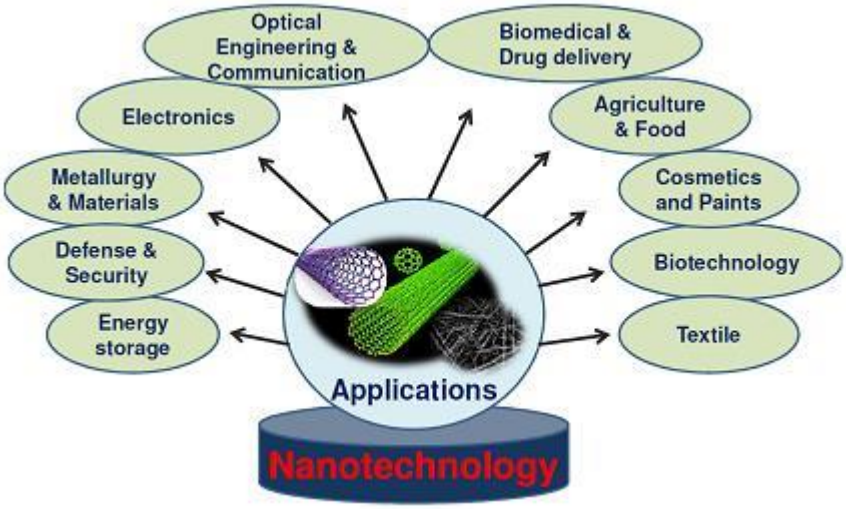


Fig. 1: Applications of Nanotechnology

1.2 Nanotechnology and Environment

Mass production of engineered nanoparticles (ENPs) may enhance their chance to interact with water, soil and air, and eventually enter the environment. Adverse impact of these ENPs on environment is still poorly known, while their use in commercial goods is frequently increasing (Jalill and Yousef, 2015).

1.2.1 Nanotechnology and Agriculture

Even though fertilizers play an important role in growth and development of plants but all of the applied fertilizers are not taken up by plants due to the processes of percolation, hydrolysis and decomposition. This nutrients loss can be controlled and crop yield can be enhanced through nanotechnology. Products of nanotechnology have the potential to release the nutrients on-demand, which can enhance plant growth and boost target activity (Nair *et al.*, 2010). This application is also essential to cope with increasing global food security and climate change challenges (Parisi *et al*., 2014). Full potential and applications of nanotechnology in the field of agriculture still need to be explored but along with it is also vital to fully understand its impacts on environment

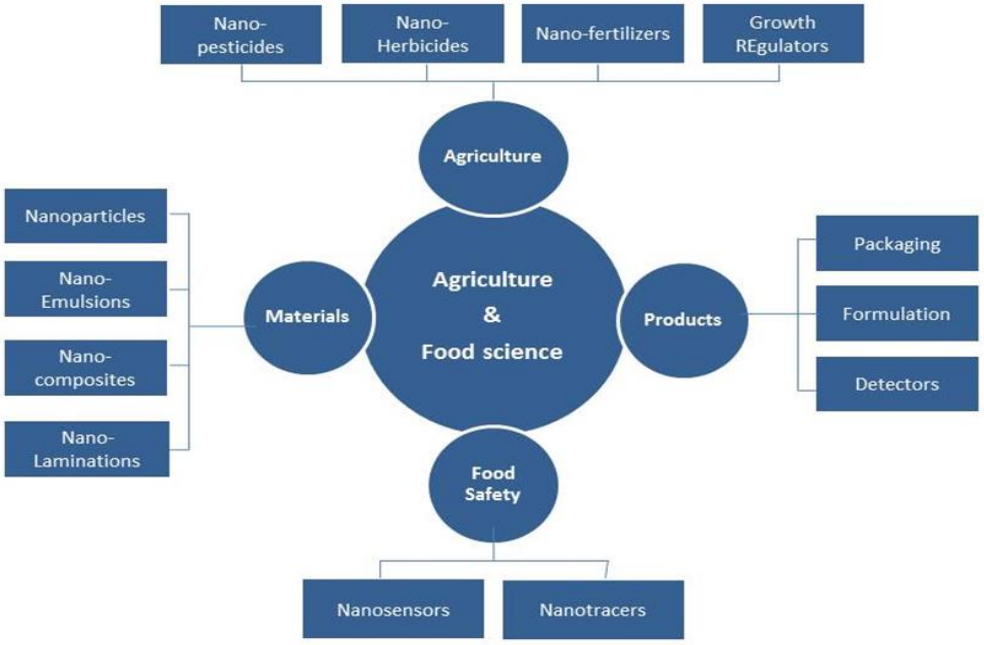


Fig. 2: Applications of Nanotechnology in Agriculture

1.2.2 Poultry Industry in Pakistan

Poultry sector is one of the most organized division of agricultural sector of Pakistan, which is playing a vital role in fulfilling protein demand by producing 26.8%

of total meat. It has a share of 5.76 and 1.26% respectively in agricultural sector and overall GDP (Hussain *et al.,* 2015; GOP, 2014). Annual meat consumption raised by 4% due to vibrant growth of poultry in 1980s (Sadiq, 2004). Nanotechnology is known for diagnosis and treatment of diseases and efficient drug delivery (Muktar *et al.,* 2015). Nanotechnology is also becoming attractive option for increasing the feed efficiency, promoting growth, increasing the feed efficiency and reducing the price of the poultry meat (Elkloub *et al.,* 2015).

1.3 Why Titanium Dioxide Nanoparticles (TNPs)?

A number of nanoparticles have already been tested for their impacts on poultry including nano silver, nano selenium, nano copper and zinc oxide nanoparticles (Ahmadi *et al.,* 2013; Pineda *et al.,* 2013). Impacts of TNPs on chicken are not explored yet. Due to wide range of production and usage of these particles there is a possibility that these can influence poultry either through direct utilization or indirect exposure, so their impacts were evaluated on chicken.

1.4 Significance of Study

A number of studies have been conducted to evaluate impacts of nanoparticles on growth of plants but little information is available on phytotoxicity induced by nanoparticles application and their accumulation in grains. This study was conducted to determine phytotoxicity in response to nanoparticles. Furthermore no study has yet investigated impacts of Titania nanoparticles on chicken, which has been done in this specific study.

1.5 Objectives of Study

On the basis of literature review, it was hypothesized that TNPs may induce phytotoxicity in rice and affect native chicken. The specific objectives of present study were:

a. Determination of growth response of rice plants upon exposure to TNPs
b. Assessment of phytotoxicity in rice due to TNPs application
c. Evaluation of toxic effects of TNPs on serum biochemical changes in chicken

1.6 Scope of Study

The scope of the study is to provide an assessment of TiO_2 nanoparticles on growth performance of rice and chicken. This study provided new insights of how nanotechnology can be used to enhance yield and its possible adverse impacts on rice and chicken.

Chapter 2

LITERATURE REVIEW

This chapter highlights applications of nanopartciles (NPs), their impacts on plants; phytotoxicity, bioaccumulation, root, shoot length, biomass, their applications in poultry and impacts on animal models in case of their entry in food chain.

2.1 Nanotechnology

The science of understanding and controlling the matter at sizes of approximately 1–100 nm is called nanotechnology. Matter at this size bears unique physical properties, which can render in various innovative applications. Nowadays nanotechnology is gaining prominent place in human life due to its unique applications in science and technology (Mukhopadhyay, 2014).

2.2 Application of Nanoparticles in Agriculture

Nanotechnology can change the entire setup of the existing agricultural and food industry by treating plant diseases, detecting pathogens and by improving the ability of plants to absorb nutrients, etc. It can also help in fighting crop pathogen with the application of Nano biosensors (Rai and Ingle, 2012). With the advancement of nanotechnology in the field of agriculture, it is essential to understand its impacts on environment and especially on crops. A number of studies have been conducted to investigate environmental threats of NPs and risk of their entry in food chain. But still the information is insufficient and further studies are required.

2.2.1 Impact of Nanoparticles (NPs) on Plants

Plants provide a major route for the entry of nanoparticles in food chain. A number of studies have been conducted to investigate influence of nanoparticles on plant through various mechanisms. Plant cell wall is a primary and major barrier that

prevents entry of nanoparticles. Only the agents of size smaller than pore size of cell wall (5-20 nm) cross cell membrane. Nanoparticles are also capable of changing morphology of root cells by enhancing pore size and increasing their uptake. A study assessed toxicity of seven metal oxide nanoparticles on maize and rice, findings showed that none of the NPs influenced seed germination. However copper oxide and zinc oxide NPs significantly suppressed root elongation at 2000 mg/L. Aluminium oxide nanoparticles showed minor toxicity. Remaining four NPs produced no noticeable impact. This study suggested that phytotoxic impact of NPs depends on concentration level (Yang *et al.*, 2015).

Silver nanoparticles (AgNano) are reported to significantly decrease biomass and induce chlorophyll retardation in *V. radiata* at 1000 μg/mL. Results also revealed breakage of cell wall and vacuoles of treated plants (Mazumdar, 2014). Another study compared phytotoxic impacts and accumulation of zinc oxide (ZnO) NPs and bulk ZnO in green pea. ZnO NPs enhanced root elongation while the bulk ZnO increased both root and stem elongation (Mukherjee *et al.*, 2014).

Nanoparticles are considered less toxic then their ions proved by a study on ZnO and silver nanoparticles (Pokhrel and Dubey, 2013). A study was conducted to investigate impacts of five different NPs (multi-walled carbon nanotube, aluminum, alumina, zinc, and zinc oxide) on six plant species (radish, rape, ryegrass, lettuce, corn, and cucumber. Results reported that only ZnO NPs influenced seed germination. The concentration level of 2000 mg/L of ZnO and Zn-Nano completely inhibited root elongation of treated plants (Lin and Xing, 2007).

2.2.2 Impact of NPs on Rice

Rice is the one of the major staple food around the world, consumed by more than half of the world's total population. It is also important from nutritional point of

view as it contains more calories than wheat and corn (Kennedy, 2002). Therefore it is important to investigate impacts of nanoparticles on yield and grain quality of rice. TNPs at concentration levels of 0.01, 1 and 10 mg mL^{-1} has no toxic effect on rice while bulk particles has no effectiveness in rice growth (Jalill and Yousef, 2015). Silver nanoparticles of size 20 nm can be trapped in root cells and are prevented from translocation while larger particles of 150 nm are translocated and cause deformation of leaf cells of rice, while Silver nanoparticles of 25 nm penetrate in root cells and damage vacuoles. Damage of cell wall was due to entrance of larger particles through small pores of cell wall (Thuesombat *et al.,* 2014; Mazumdar and Ahmed, 2011).

A study evaluated effect of aluminium on morphology and cell structure of root cells in rice and proved that toxic levels of aluminium induce structural changes in root cells (Alvarez *et al.,* 2012). In another study, toxicity of ZnO and TNPs on rice seed germination were tested. Reduction in seed germination and number of roots was shown by ZnO nanoparticles while root length was also inhibited by ZnO NPs. Whereas TNPs found to have no impact on length of roots (Boonyanitipong *et al.,* 2011).

2.2.3 Accumulation of NPs

Nanoparticles can enter in root cells either due to their small size or by rupturing pores of cell wall. As nanoparticles translocate to aerial parts of plants they not only influence plant growth but also accumulate in plant tissues and in edible parts. A study analyzed impact of cerium nanoparticles on quality of rice grains. Medium and low-amylose varieties showed more accumulation of cerium nanoparticles in grains than the high-amylose variety. These results suggested that $nCeO_2$ could negatively impact the quality of rice (Rico *et al.*, 2013).

TNPs were found to enhance barley kernel yield at 1000 mg kg^{-1} concentration level, with enhanced Ti levels accumulated in grains (Poscic *et al.,* 2016). Higher concentration of zinc was also found in soybean pods when grown in zinc oxide nanoparticles amended soil (Hernandez-Viezcas *et al.,* 2013). Zinc oxide nanoparticles accumulate in plant tissues along with significant decrease in plant biomass up to 1000 mg/L (Rao and Shekhawat, 2014). Lettuce is one of the ten plants recommended for phytotoxicity evaluation by US EPA (1996). Studies showed that cerium oxide nanoparticles containing soil increases levels of Ce in roots and higher concentrations also significantly translocate and accumulate in shoots (Gui *et al.,* 2015).

Larue *et al.* (2012) studied the response of wheat to TNPs with a diameter of 12-140 nm. They indicated that smallest TNPs were accumulated in roots and then translocated to upper part of plants. Above 36 nm NPs are accumulated in root parenchyma and cannot be translocated. They suggested a threshold diameter of 36 and 140 nm. Above these levels, accumulation can be minimized.

2.3 Titanium Dioxide Nanoparticles

Titanium dioxide (TiO_2) is a fine, white, crystalline, odorless, low-solubility powder which was considered to be less toxic. It exists in three natural forms of rutile, anatase, and brookite. It has good fatigue strength, resistance to corrosion, biocompatibility, whitening and photocatalysis, as well as excellent optical performance and electrical properties. Due to its excellent physicochemical properties, it is a widely being used in industrial sector. It has been reported that titania nanoparticles (TNPs) increase crop yield by 30 % by promoting plant growth and alleviating disease severity (Jalill and Yousef, 2015; Zhang *et al.,* 2012; Iavicoli *et al.,* 2012).

According to a report 40,000 tons of TNPs were produced in America during 2006 and due to ever-increasing market demand, it is expected that its production will be increased to 2.5 million tons per year by 2025. This over production and over usage will lead to a significant release of TNPs into the environment (Zhang *et al.*, 2012). A number of studies have been conducted to investigate interaction of TNPs with plants, their impact on growth and crop yield.

2.3.1 Impact of Titania Nanoparticles (TNPs) on Plants

A study compared impacts of iron oxide nanoparticles and titania nanopartilces (TNPs) on plant performance and rhizobial microbes in soybean plants. Results showed that both NPs have different effects on plant growth and root microbes. Furthermore charge on NPs plays a significant role on type of impact (Burke *et al.*, 2015). Lower concentrations of TNPs have a positive impact on seed germination and growth in onion while higher concentrations inhibits growth promotion (Laware and Raskar, 2014). Another study found that TNPs have significant effect on root, shoot length and biomass in all concentration levels. They increase root, shoot length and biomass of wheat up to 60 mg kg^{-1} concentration level. Higher concentrations inhibit plant growth (Rafique *et al.*, 2014).

A study investigated impact of TNPs on seed germination of wheat. Findings indicated that higher concentration of TNPs i.e. 400 mg L^{-1} has negative impact on plant growth (Sana Ullah and Arshad, 2014). Spraying of TNPs is found to have no detrimental effect on root, shoot length while it decreases plant biomass (Mahmoodzadeh *et al.*, 2013). TNPs also enhance photosynthesis by activating photochemical reactions of chloroplasts in spinach (Hong *et al.*, 2005).

2.3.2 Impacts of TNPs on Animal Models

Because of extensive environmental exposure to TNPs, they can easily enter human body through various routes like oral, inhalation, and dermal route while oral exposure can occur regularly through consumption of food (Zhang *et al.*, 2012; Adeyemi and Adewumi, 2014; Geraets *et al.*, 2014). As liver detoxifies the body it is the most susceptible target organs of NPs (Vasantharaja *et al.,* 2014). Several studies have shown toxic effects of TNPs on liver functions (Iavicoli *et al.*, 2012), but still information regarding magnitude of NPs released and exposed to organisms is insufficient (Alkaladi *et al.*, 2015). Impact of nanoparticles on human is investigated using various rodent models through various exposure routes and conditions.

2.3.2.1 Impact of Nanoparticles on Rats

A number of studies have been conducted to evaluate potential health impacts of TNPs and several studies consider small amount of TNPs as biologically inert and nontoxic for animals and humans. High doses and prolonged exposure of TNPs have been reported to induce pulmonary inflammation, fibrosis, epithelial hyperplasia, and tumorigenesis in animals (NIOSH, 2011).

Vasantharaja *et al.* (2015) studied toxicity of titania nanoparticles (TNPs) on serum biochemical changes in male Wistar rats through oral exposure with two different doses of 50 and 100 mg kg^{-1} body weight (BW). It was concluded that liver and kidneys were significantly damaged in both treatment groups. To investigate the cardiotoxic effects of TNPs, two different doses of TNPs were given along with two natural antioxidants i.e. carnosine and melatonin. Results of the study suggested that toxic effect of TNPs on heart tissues of rats depend on dose and carnitine or melatonin that effectively reduce toxic effects of low dose TNPs (Al-Rasheed *et al.,* 2015).

Another study reported toxic effects of TNPs on liver in Wistar rats, showing significant changes in ALP and ALT levels and damage of liver tissues (Fatemeh and Mohammad, 2014). Tissue distribution and elimination of different TNPs through oral and intravascular route of exposure was evaluated. Results suggest that low oral bioavailability and slow elimination in oral administration can lead to long term accumulation in tissues (Geraets *et al.,* 2014). In another study determined impact of TNPs on immune system of rates. Findings showed an increase in number of B cells in blood stream, thus TNPs trigger immune system response (Fu *et al.,* 2014).

Oral administration of TNPs at different doses of 0.16, 0.4 and 1 g kg^{-1} BW of rats are capable of slight liver and heart injury as well as disturbance in amino acid metabolism (Bu *et al.,* 2010). In order to assess pulmonary toxicity of TNPs rats were intra-tracheally instilled with three different forms and different concentration of TNPs i.e. 0.5, 5, or 50 mg kg^{-1} of 5, 21, and 50 nm TiO_2 primary particles. Results showed that pulmonary toxicity depends on particle size and exposure dose (Liu *et al.,* 2009). Another study was conducted to determine impact of different sized TNPs on renal and hepatic functions and correlate it with oxidative stress in rats through intra-tracheal instillation. The results of study provided no significant proof that small sized particles produce more toxicity than large sized particles (Liang *et al.,* 2009).

2.3.2.2 Impact of Nanoparticles on Mice

Impact of TNPs on endocrine in mice was determined through oral administration of 0, 64 and 320 mg kg^{-1} of TNPs. Study concluded that these concentrations of TNPs increase plasma glucose level and insulin resistance (IR) by elevating reactive oxygen species (ROS) (Hu *et al.,* 2015). Another study evaluated liver injury induced by TNPs in mice. Different concentrations of TNPs i.e. 5, 10, 50, 100, and 150 mg kg^{-1} BW and 150 mg kg^{-1} BW of bulk titanium dioxide (TiO_2) were

injected in abdominal cavity of mice. Results showed TNPs accumulation in liver DNA and liver dysfunction (Ma *et al.,* 2009). TNPs cause clastogenicity, genotoxicity, oxidative DNA damage, and inflammation in mice only after 5 days of exposure through drinking water. These results describe TNPs as carcinogenic and mutagenic chemical (Trouiller *et al.*, 2009).

2.4 Role of Nanoparticles in Poultry

Nanotechnology is also becoming attractive option for increasing the feed efficiency, promoting growth, controlling diseases, targeting drug delivery, increasing the feed efficiency and reducing the price of the poultry meat (Elkloub *et al.*, 2015). Some of products of nanotechnology which have been investigated as nutrients additives in poultry include nano silver, nano selenium, nano copper and zinc oxide nanoparticles (Ahmadi *et al.*, 2013; Pineda *et al*., 2013).

A study reported that feeding of calcium phosphate nanoparticles as a source of calcium and phosphorus minerals increases the feed efficiency among broiler chickens. Results of this study showed that chicken fed with 50 and 60 % of calcium phosphate NPs had higher weight gain than the control group (Vijayakumar and Balakrishnan, 2014). Another study suggested that copper nanoparticles (CuNano) increased levels of RBC, HGB, HTC, heterophils, monocytes and basophils. They also elevated levels of calcium, phosphorus and iron and decreased levels of glucose and cholesterol (Mroczek-Sosnowska *et al.,* 2013). When platinum nanoparticles were injected in ovo to determine their impact on embryo development and brain morphology, findings suggest that NP-Pt reduce number of proliferating cells in the brain tissue. However they has no influence on growth and development of embryo (Prasek *et al.,* 2013). Injected copper nanoparticles (CuNano) in fertilized chicken

eggs indicated depressed development of embryo and alteration in metabolic rate (Pineda *et al.,* 2013).

Nanoparticles of chromium picolinate are found to improve retention of iron, zinc and calcium as well as increase chromium and calcium in liver and number of lymphocytes in broiler chicken (Sirirat *et al.,* 2012). Sawosz *et al.* (2012) compared impacts of AgNano, glutamine (Glu) and the complex of nanoparticles of silver and glutamine (Nano-Ag/Glu) on muscle development of broiler embryos. Findings indicated that all three treatments do not depress development. However AgNano and Nano-Ag/Glu increase number of nuclei per cell while Nano-Ag/Glu also has positive impact on muscles.

Another study investigated impact of AgNano on gene expression in breast muscle and heart in growing chicken. Results revealed significant changes in the expression of FGF2 and VEGFA genes on the mRNA and protein levels in growing chicken (Hotowy *et al.,* 2012). AgNano also influence nitrogen utilization and immunoglobulin (1gG) in seven days old broilers but they have no effect on energy metabolism, growth performance and microbial population. AgNano treated chicken show decreased immunity and oxidative stress (Pineda *et al.,* 2012; Ahmadi and Kurdestany, 2010). In another study, AgNano were injected in fertilized chicken eggs to determine their impact on development of embryo. Results showed that silver nanoparticles has no effect on embryo development but they decrease number and size of lymph follicles (Grodzik and Sawosz, 2006).

2.5 Biochemical Blood Profile of Chicken

While comparing blood biochemical profile of three different strains of male and female chicken it was found that all strains (three ecotypes) had significantly different values for plasma total protein, inorganic phosphorus, uric acid, sodium and

potassium. However, the values of albumin, calcium and cholesterol were almost same in three ecotypes. Levels of uric acid and potassium varied depending upon sex of chicken while other parameters were not sex dependent (Elagib *et al.,* 2012).

A study investigated impact of dietary supplementation of rosemary and rosemary volatile oil on serum variables of broiler chicken. Results indicated an increase in serum superoxide dismutase activity (SOD) and enhanced oxidation mechanism. This study also assumes that rosemary plant causes hypocholesterolemic effect (Polat *et al.,* 2011). Aflatoxin in feed induces liver dys-functioning in broiler chicken. It increases levels of alanine aminotransferase (ALT), aspartate aminotrans-ferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBILI) significantly within 24 to 48 hours of administration (Rizvi and Shakoori, 2000).

2.6 Related Research Work at IESE, NUST

Lettuce was grown in soil amended with different concentrations (0, 25, 50, 75 and 100 mg kg^{-1}) of TNPs. Results showed an increase in root/shoot length by 1.5 fold and plant biomass by 2 folds as compared to control (Hanif *et al.,* 2015). Another study assessed toxicity of TNPs on wheat in hydroponic culture with concentration levels of 0, 25, 50, 100, 200, 400 and 600 mg kg^{-1}. Maximum root elongation was observed at 200 mg kg^{-1} while higher concentrations inhibit length. No significant toxic effect was found on short term exposure (Sana-Ullah and Arsad, 2014).

Another experiment was conducted to determine impact of TNPs (0, 20, 40, 60, 80, 100 mg kg^{-1}) on wheat plant. Root, shoot length and plant biomass was significantly affected by TNPs treatments. Plant length and biomass increased up to 60 mg kg^{-1} while higher concentrations not only decreased length but also reduced biomass (Rafique *et al.,* 2014).

In another study lettuce was exposed to TNPs treatments (0, 50, 100, 150, 200 and 250 mg kg^{-1}) for 90 days in soil medium. Shoot length increased by 36% while biomass was increased to 1.4 fold as compared to control (Zahra *et al.*, 2015).

From above cited literature and studies conducted at IESE, it was hypothesized that TNPs can affect rice growth and biochemical blood parameters of local chicken. To test this hypothesis, the adopted methodology is discussed in next chapter.

Chapter 3

MATERIALS AND METHODS

This section outlines all the procedures that were used to prepare TNPs, evaluate their impacts on vegetative traits and chicken, determine their accumulation in grains and damage to roots. All these methods were carried out at Environmental Biotechnology Lab of Institute of Environmental Sciences and Engineering, School of Civil and Environmental Engineering, National University of Science and Technology, Islamabad Pakistan.

3.1 Reagents used for Different Procedures

Table 1: Reagents used for Experimentation

Step	Required Reagents
Preparation of TNPs	Titanium dioxide powder
Determination of titania in grains	Nitric acid (HNO_3) Hydrogen peroxide (H_2O_2)
Treatment of animals	TNPs Chloroform

3.2 Preparation and Characterization of Nanoparticles

3.2.1 Preparation of TNPs

TNPs were prepared through liquid impregnation method. Sixty grams of weighed titania was added in 900 mL of distilled water and placed on stirrer for 48 hours. Then solution was allowed to settle for 12 hours. After settling, it was dried at 105 °C for 48 hours in oven. The dried solid was crushed in mortar and pestle and calcinated at 500 °C for 5 hours (Zeb *et al.,* 2010).

3.2.2 Characterization of TNPs

3.2.2.1 X-ray Diffraction (XRD)

X-ray diffraction was used to characterize the crystal structure of prepared TNPs and average crystal size was calculated through Scherrer's calculator using X'Pert Highscore. The XRD pattern of TiO_2 was obtained using STOE, Scintag Theta-Theta X-ray Diffractometer model with CuKα radiation in the 2θ scan range of 20°-80° (λ = 0.154) with a step of 0.5°.

3.2.2.2 Scanning Electron Microscopy

The surface morphology of TNPs was determined through SEM (JSM- 6490A, JEOL) with an accelerating voltage of 20 kV. Before scanning, the powdered TNPs were diluted 100 fold in distilled water and then sonicated for 60 minutes. A drop of 10 µL diluted solution was placed on a glass slide and air dried. The dry powder was sputter coated with gold in order to increase conductivity of surface. The coating of TNPs was done using Atomic Ion Sputtering Device, JEOL, JFC-1500, Gold 250A°.

3.2.2.3 Energy Dispersive X-ray Spectroscopy (EDX)

EDX is a chemical microanalysis technique used in conjunction with SEM to illustrate the elemental composition of sample. EDX spectra were generated for TNPs to determine their composition.

3.3 Soil Preparation

Clayey soil was taken from National University of Science and Technology (NUST), H-12 sector, Islamabad, Pakistan. This soil was air dried for 4 days and after removal of moisture it was ground in ball mill and sieved in sieve shaker to get homogenous fine soil. Sand was purchased from local nursery of Islamabad and after air dried this sand was mixed with soil in the ratio of 1:3 respectively, to make the texture of soil suitable for rice cultivation.

3.4 Preparation of Pots

1250 grams of soil was weighed for each plastic pot with proper labeling. TNPs suspensions of five concentrations i.e. 500, 600, 700, 800, 900 and 1000 mg/kg were prepared. For each concentration level there were eight replicates. Beside these concentrations there was also a control group in which nanoparticles were not added, for comparison with treated ones. Pots were filled with water and mixed well three to four times before seedlings transplantation.

3.5 Seedlings Transplantation

25 days old seedlings were purchased from Gujrat. Roots and shoots of seedlings were washed with distilled water to remove soil and ensure clarity. Seedlings were shifted to the pots containing soil amended with TNPs and filled with water as required for rice cultivation. In each pot three seedlings were planted.

3.6 Chlorophyll Content Measurement

Chlorophyll content of rice plants grown in greenhouse was measured three times with 14 days of interval. Measurements were taken from thirty points of each plant using portable chlorophyll absorbance meter (CCM-200 Plus). Chlorophyll meter weighs 168 g with a 0.73 cm^3 measurement area and calculates chlorophyll content index (CCI) based on the absorbance measurements. Whole plant leaf was used for estimation of chlorophyll content.

3.7 Determination of Impact of TNPs on Rice Plants

Impacts of TNPs on rice were determined after harvesting. Morphological parameters like root and shoot lengths, fresh and dry weight were measured. After harvesting of rice plants, roots and shoots were washed with distilled water and their lengths were measured separately.Roots and shoots of rice were cut and oven dried

by keeping them in oven at 80° for 24 hours in aluminum foils. After drying root and biomass were measured separately. Grains were then stored for Titanium dioxide determination.

3.8 Phytotoxicity Evaluation

Phytotoxicity was determined in roots through SEM analysis and in grains through Ti detection. For phytotoxicity evaluation in rice roots, seeds were germinated for seven days with TNPs exposure then samples were oven dried at 60 °C for three hours. The samples were allowed to cool at room temperature. A fine piece of root was then observed under Scanning Electron Microscopy (SEM).

For analysis of TiO_2 200 mg of ripe grains of were ground separately and digested in 5 mL of 65% HNO_3. After heating at 120°C for 90 min it was cooled down. 3 mL of 35% H_2O_2 was added to the samples and left for overnight. In the morning the solutions were carefully heated until solutions were completely clear. The solutions were transferred to a 25 mL volumetric flask and 4% nitric acid was added to make the volume up to the mark. This solution was analyzed on Inductively Coupled Plasma−Optical Emission Spectroscopy (ICP) for titania estimation in grains (Rico *et al.,* 2013).

3.9 Experimental Animals

3.9.1 Treatment of Animals

A total of 30 one-day-old local chicken with average weight 27.5 g were bought from hatcheries of Poultry Research Institute (PRI), Rawalpindi. Birds were randomly assigned to five groups with six birds per group. Five TNPs concentrations (0, 10, 20, 40, 80 mg kg^{-1}) were mixed with their crushed feed to form pellets and each chick was served with one pellet daily while control group was treated with normal feed only. Birds were also served with diuretics twice a week.

3.9.2 Animals' Management

Birds were kept in properly ventilated cages placed in Animal House (National University of science and Technology, Islamabad). Cages were equipped with electric bulbs for heating. Birds were provided with free access to commercial feed and clean drinking water. After acclimatization period birds were fed with pellets of feed mixed with TNPs for 42 days.

The body weight (BW) and feed consumption of the birds were recorded daily with a calibrated balance. The average BW gain was calculated from the initial and final weights of the birds.

3.9.3 Serum Biochemical Analysis

After 42 days blood samples of chicks were collected from brachial vein and placed in 3-mL-heparinized test tubes. Collected blood samples were centrifuged (Rotofix 32a) to separate serum part and used for analysis like total protein (TP), Albumin (ALB), Cholesterol (CHOL), High density lipoprotein (HDL). Alanine amino-transferase (ALT), Alkaline phosphatase (ALP) were estimated for liver function tests. Blood urea nitrogen (BUN) and Creatinine (CREA) levels were measured for renal function test using auto analyzer (Hitachi modular P800, Hitachi Ltd, Japan).

3.10 Statistical Analysis

Data obtained from the study was subjected to statistical analysis by computing mean and standard deviation. Multi group analysis was done using one-way analysis of variance (ANOVA). T-test was performed in order to estimate differences between control group and treated groups. Statistical significance was considered at $p<0.05$.

Chapter 4

RESULTS AND DISCUSSION

4.1 Characterization of TNP

4.1.1 XRD Results of TNPs

The phase composition, crystal structure and crystallite size of TiO_2 nanoparticles synthesized by liquid impregnation method were determined through XRD.

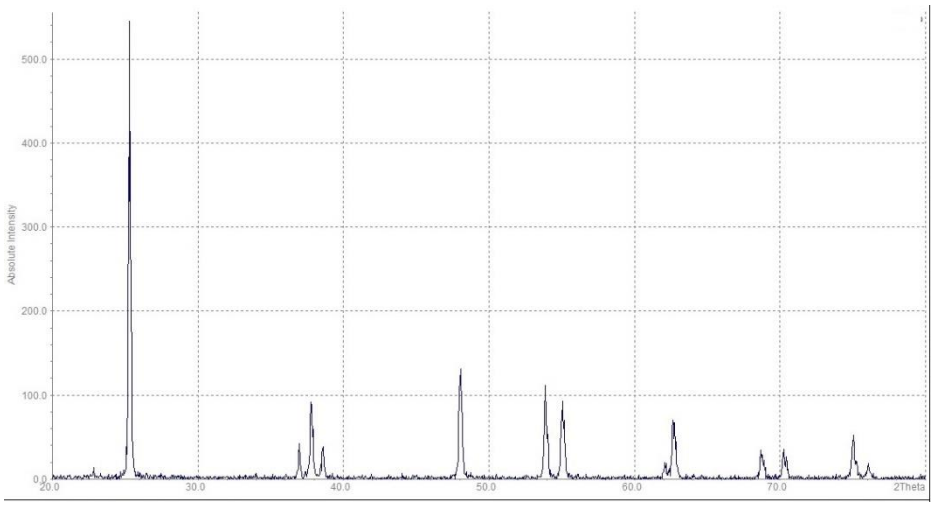


Fig. 3: XRD Spectrum of TiO_2 Nanoparticles

The spectrum in figure 3 indicates that TiO_2 nanoparticles were crystalline and no amorphous phase was observed. The first peak between 20 and 30 θ indicates the presence of anatase phase. It has been proved that Titania in anatase form exhibits significantly better photocatalytic results and reactivity than rutile and brookite (Yao *et al.*, 2006).

Besides this, anatase phase is also a powerful oxidizing agent having other characteristics like nontoxicity and long term photo-catalytic ability (Hoffmann *et al.*, 1995). Moreover, it has been reported that nanopartices when calcined at high

temperature (almost 400ºC) are converted to anatase phase having two to three time more photo-catalytic ability than the commercially prepared TNPs (Sekino, 2010).

4.1.2 SEM Results of TNPs

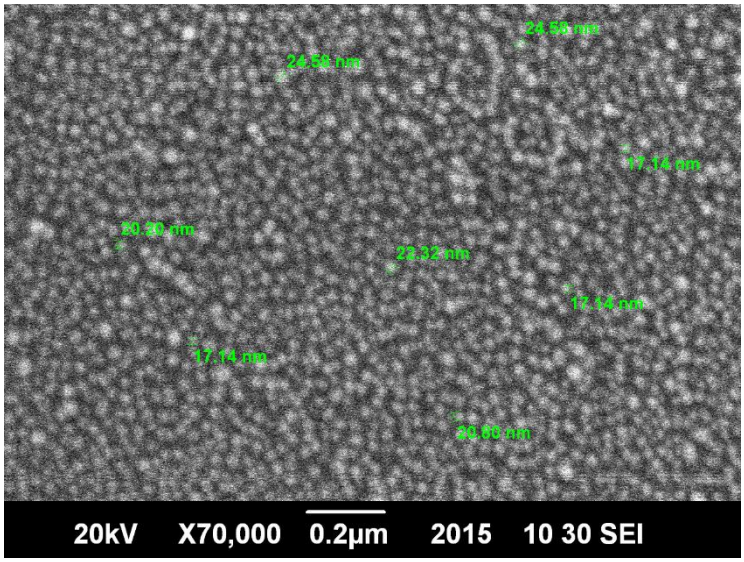


Fig. 4: SEM Image of TiO_2 Nanoparticles

Figure 4 shows the image of TNPs at 70,000 magnification (SEM, JEOL JSM-6490 A, Japan). Image confirms the size of these particles in nano range i.e. 17-30 nm.

4.1.3 EDX Results of TNPs

Energy dispersive X-ray Spectroscopy is used for elemental analysis of samples. Figure 5 represents the EDX spectra of TNPs, indicating the presence of pure TiO_2. The first red peak indicates presence of carbon which is result of carbon tape used while the middle red peak is of gold which was used for coating during EDX analysis. Remaining peaks show presence of titanium and oxygen.

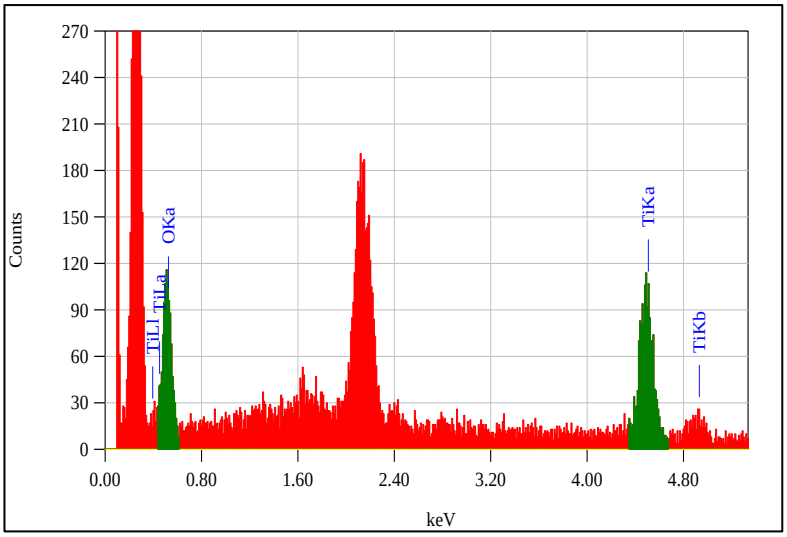


Fig. 5: EDX Spectra of TiO_2 Nanoparticles

4.2 Growth Response of Rice to Nanoparticles

4.2.1 Root and Shoot length

Figure 6 is showing impact of TNPs on shoot length and it indicates that maximum length had been achieved at 700 mg kg^{-1} treatment level. At this level shoot length had shown a significant increase of 20% as compared to control.

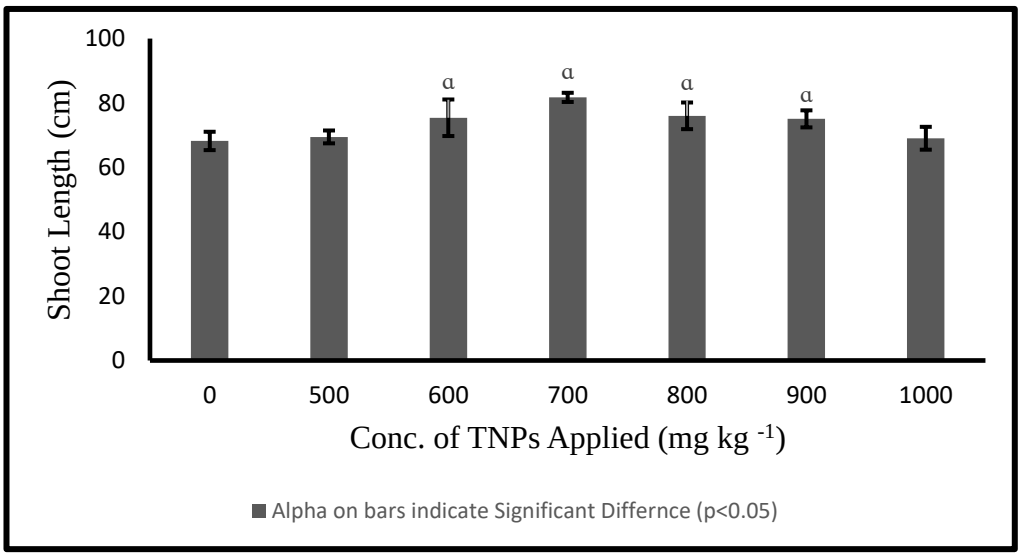


Fig. 6: Shoot Length of Rice in Response to TNPs Treatment

Lower concentrations of TNPs (0.01, 1 and 10 mg mL^{-1}) have been reported to induce no significant effects in root and shoot length of rice (Jalill and Yousaf 2015). Another study reported an increase of 36% in shoot length of TNPs treated wheat (250 mg kg^{-1}) as compared to control (Zahra *et al.*, 2015).

Root length was increased with increase in concentration of TNPs up to 600 mg kg^{-1} and afterwards it again started decreasing as shown in figure 7. At 600 mg kg^{-1} root length had shown an increase of 31% as compared to the control group. While at 1000 mg kg^{-1}, root length was only 7% greater than control group.

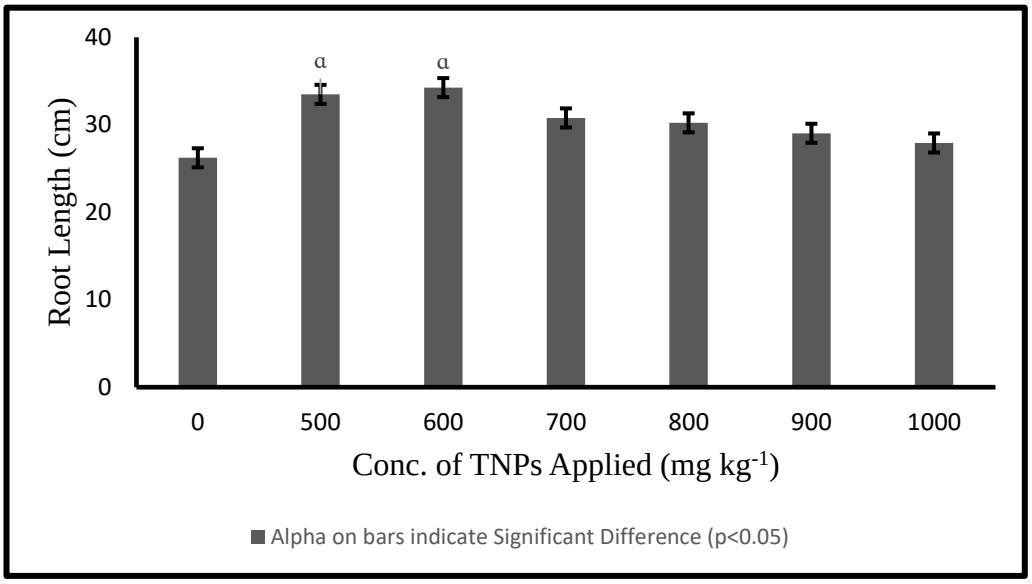


Fig. 7: Root Length of Rice in Response to TNPs Treatment

While another study reported an increase of more than 50 % in root elongation in wheat at 50 and 100 mg L^{-1} of TNPs as compared to control (Larue *et al.*, 2012). 100 mg L^{-1} of TNPs improve root and shoot length of wheat while at higher concentrations root and shoot length decreases (Mahmoodzadeh *et al.*, 2013). *Mentha piperita* showed improved root length at 100 mg L^{-1} of TNPs while higher concentrations had inhibitory effects on root length. This positive effect of TNPs is

attributed to antimicrobial properties that enhanced plant resistance to stress (Samadi *et al.,* 2014).

4.2.2 Plant Biomass

In accordance with root length, root biomass was also maximum at 600 mg kg^{-1} (fig 8). While highest concentration of 1000 mg kg^{-1} had 51% decreased biomass as compared to 600 mg kg^{-1} concentration level. As compared to control, plants treated with TNPs had shown improvements in biomass by 10% at 600 mg kg^{-1} of TNPs.

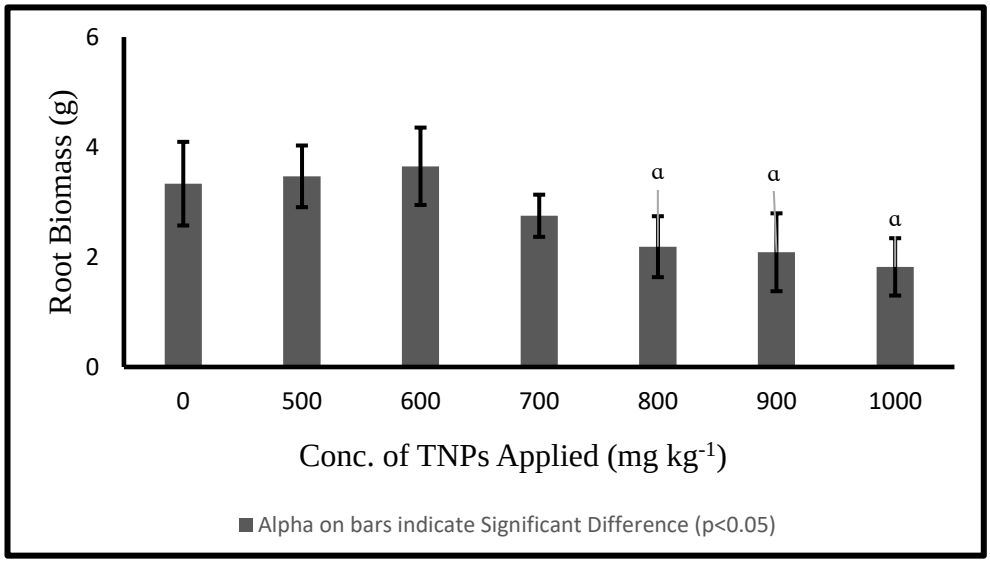


Fig. 8: Root Biomass of Rice in Response to TNPs Treatment

In another study 0.01, 1 and 10 mg mL of TNPs are reported to have no significant effect on rice biomass (Jalill and Yousaf 2015). TNPs resulted in decreased growth of soya beans (Burke *et al.,* 2015). TNPs treated wheat has shown increased root, shoot length and biomass up to 60 mg kg^{-1} concentration while growth decreases at higher concentrations (Rafique *et al.,* 2014).

Shoot biomass was showing an increase with increase in concentration levels of TNPs (fig 9). This increase was up to 800 mg kg^{-1} and at 900 and 1000 mg kg^{-1} it was showing a decrease. At 800 mg kg^{-1} maximum biomass was 21% improved as compared to control.

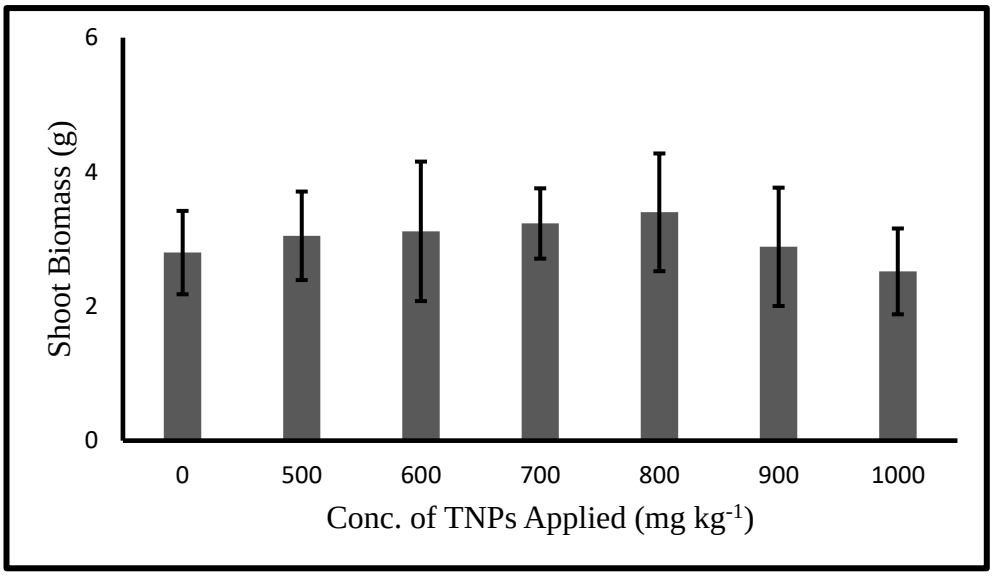


Fig. 9: Shoot Biomass of Rice in Response to TNPs Treatment

Another study reported a decrease of 9 % in root biomass, 11 % in shoot length and 23 % in root length at 1000 ppm TNPs exposure in rice. Dry root biomass increase by 476% at higher concentration of TNPs, which is referred to significant accumulation of TNPs in roots at 100 and 1000 ppm (Da Costa and Sharma, 2015). 0.02 % of TNPs is observed to have positive impacts on all vegetative traits of wheat (Jaberzadeh *et al.,* 2013). Higher concentrations of zinc oxide nanoparticles (1000, 1500 mg L^{-1}) are also reported to limit biomass in *Brassica juncea* (Rao and Shekhawat, 2014).

TNPs smaller than 140 nm can pass through root epidermis of wheat while in order to cross casparian band and translocate to shoot, primary diameter of particles should be smaller than 36 nm (Larue *et al.,* 2012). TNPs controls enzymes of nitrogen metabolism i.e. nitrate reductase, glutamate dehydrogenase, glutamine synthase, and glutamic-pyruvic transaminase that promotes nitrate absorption leading to enhanced biomass (Yang *et al.,* 2006; Mishra *et al.,* 2014; Rao and Shekhawat, 2014).

4.2.3 Number of Spikes

Figure 10 represents relation of spikes with TNPs. Number of spikes decreased at 500 mg kg^{-1} then it showed an increase with increase in concentration levels up to 900 mg kg^{-1} and then again decreased at 1000 mg kg^{-1}. 800 and 900 mg kg^{-1} had maximum number of spikes.

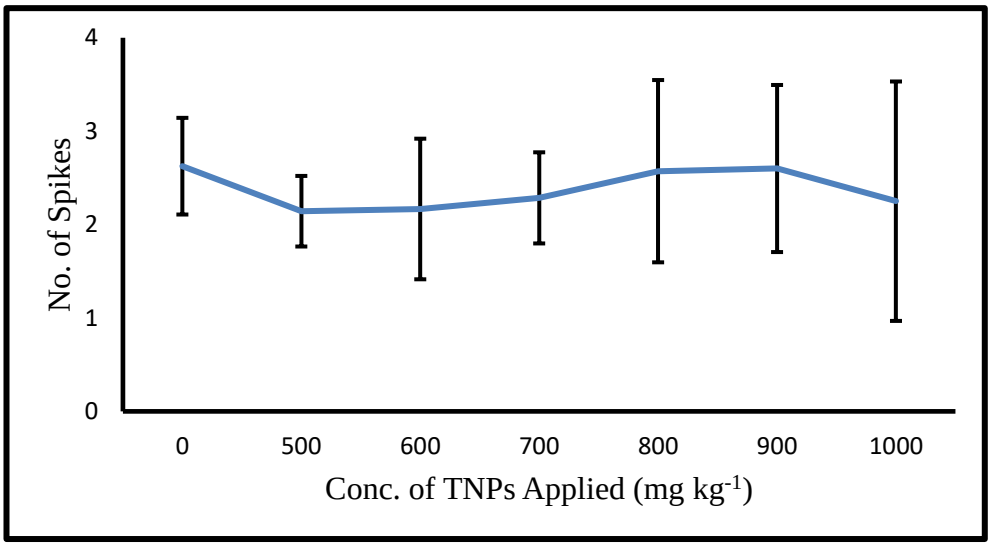


Fig. 10: No. of Spikes of Rice in Response to TNPs Treatment

TNPs at 0.04% had been proved for highest grain yield of Safflower (Morteza *et al.*, 2015). Another study reported 1000 mg kg^{-1}concentration of TNPs beneficial in barley yield (Poscic *et al.*, 2016). Silver nanoparticles at 25 and 50 ppm have highly promising impacts on grain yield of wheat (Razzaq *et al.*, 2016).

4.2.4 Chlorophyll Content

Figure 11 represents trend of chlorophyll against different concentrations of TNPs at three different ages of rice plants. At the age of 54 days plants had maximum chlorophyll content which again decreased on 69th day because leaves start turning brown. First reading was taken when plants were 40 days old in which 800 mg kg^{-1} treated plants were showing 29 % enhanced chlorophyll content as compared to control. Difference among all treatments and control group was not significant. On

54th day 800 mg kg^{-1} had 44% ($p<0.05$) improved chlorophyll content as compared to control. While on 69th day 800 mg kg^{-1} was showing a significant increase ($p<0.05$) of 42% in chlorophyll content than control group. Therefore 800 mg kg^{-1} was the optimum concentration of TNPs for maximum chlorophyll content in rice.

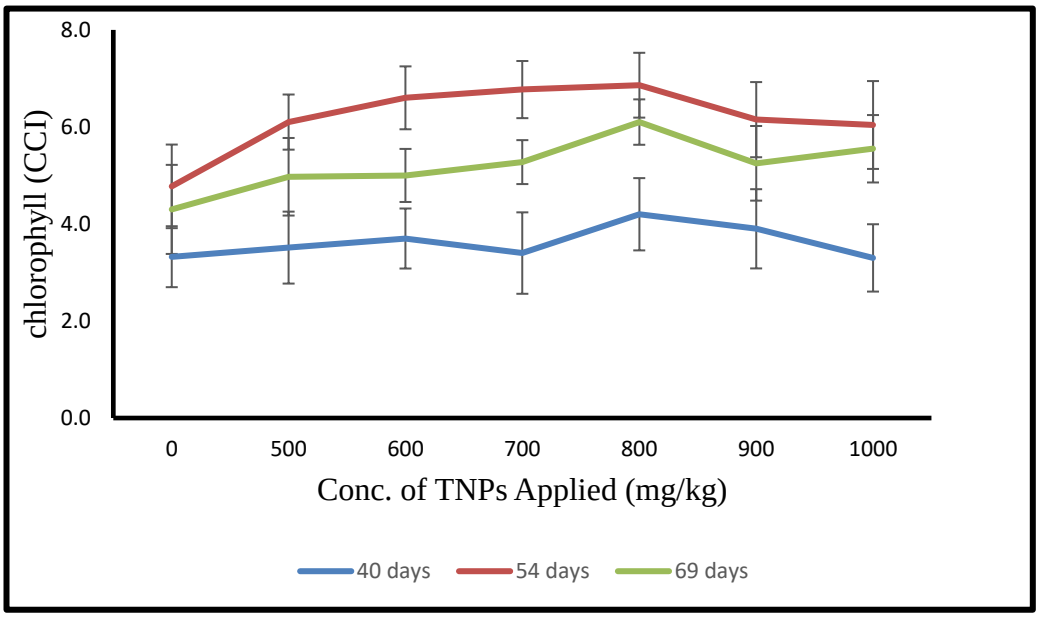


Fig. 11: Chlorophyll Content in Rice at Different Ages in Response to TNPs

It was studied that 100 mg L^{-1} of TNPs significantly increases ($p<0.05$) chlorophyll content of *Mentha piperita* as compared to control. TNPs are also known for improving the structure of chlorophyll which leads to better capture of sunlight. Studies have shown that TNPs can enhance rate of photosynthesis, chlorophyll formation and nitrogen metabolism at an optimum concentration (Samadi *et al.*, 2014). TNPs promote chlorophyll formation by stimulating Ribulose 1, 5-bisphosphate carboxylase (Rubisco) activity, which improves photosynthesis and plant development. It is also suggested that TNPs protects chloroplast from excessive light by boosting the activity of antioxidant enzymes (Siddiqui *et al.*, 2015).

4.3 Phytotoxicity Evaluation

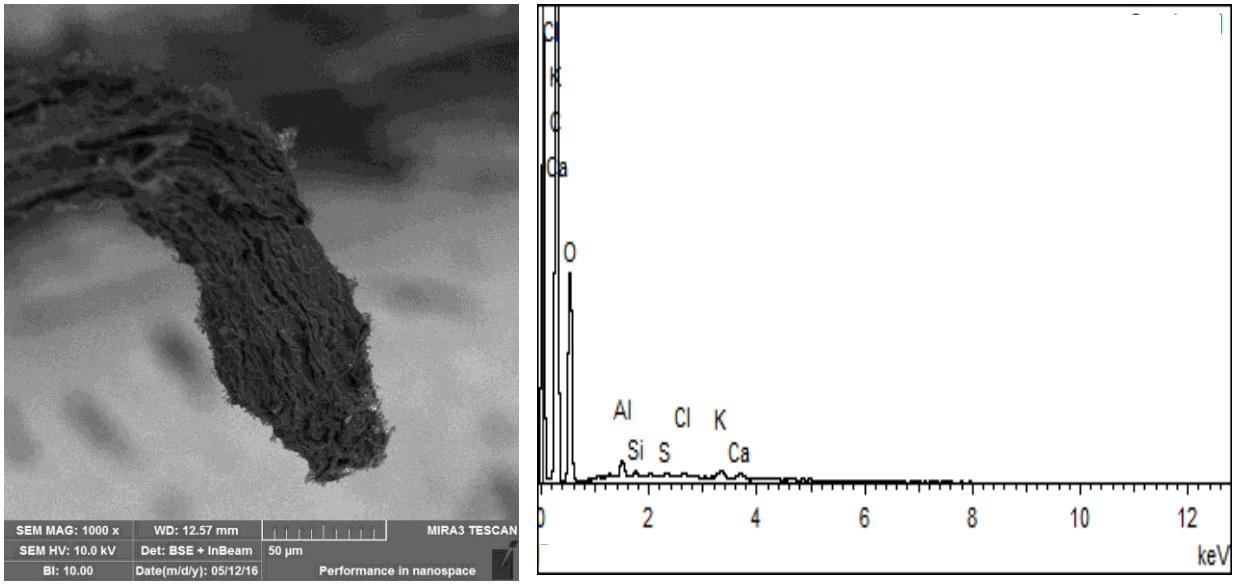
Phytotoxicity was determined in grains and roots by using different spectroscopic techniques.

4.3.1 Titanium Accumulation in Grains

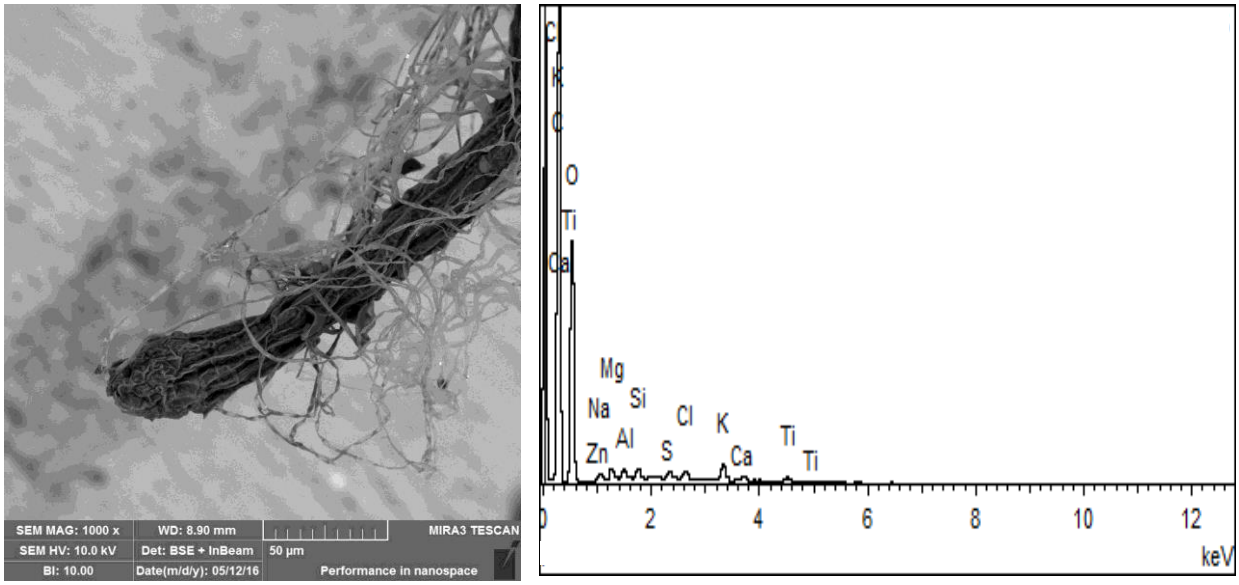
No Ti was detected in rice grains, this may be because of large size of nanoparticles (30 nm) which have already been reported to be not taken up by roots in maize due to smaller pore size of root cell wall (Asli and Neumann, 2009). Contrary to our study cerium oxide nanoparticles (8 nm) and TNPs (24 nm) accumulation are reported in rice grains and barley kernels respectively (Rico *et al.,* 2013; Poscic *et al.,* 2016). Cerium oxide nanoparticles are found to significantly enhance Ce accumulation in tomato fruits, soybean pods and lettuce (Wang *et al.,* 2012; Priester *et al.,* 2012; Gui *et al.,* 2015). In another study Ti was also detected in wheat grains, but the difference was not significant between control and treated samples (Klingenfuss, 2014).

4.3.2 Phytotoxicity Evaluation

Figure 12 shows SEM images of rice roots taken at 1000X. The control group (Fig 12A) illustrated clear surface as compared to the other treated group. Extensive root hair are visible in Fig 12B. This means that TNPs have increased root development. The elemental analysis (EDX) has also identified TiO_2 in treated group. As root hair increase surface area in contact with soil so there might be the reason that extensive root system have allowed maximum nutrients uptake which lead to enhanced plant growth in TiO_2 nanoparticles treated plants as compared to control.



(A) SEM image & EDX Spectra of Rice Root (control)



(B) SEM image & EDX Spectra of Rice Root (TiO_2)

Fig. 12: SEM image and EDX spectra of Rice Roots

In another study SEM images revealed no significant effect of silver nanoparticles on morphology and anatomy of *Bacopa monnieri* when compared to the control and treated plants (Krishnaraj *et al.*, 2012). Contrary to our results silver nanoparticles induced distortion of root epidermis with reduction of root hairs in sunflower (Krizkova *et al.*, 2008).

The morphological changes in the rapeseed roots induced by microsized and nanosized molybdenum octahedral clusters were also reported in another study. This was confirmed by SEM that microclusters have enhanced root production while nanoclusters inhibited growth by severely damaging root epidermis (Aubert *et al.*, 2012).

4.4 Effect of Titania Nanoparticles on Biochemical Blood Profile

4.4.1 Total Protein

Figure 13 shows impact of different doses of TNPs on total protein levels in blood sera. Increase in doses resulted in no significant change in levels of total protein. A decrease of 4, 5 and 6% was observed in 10, 20 and 80 mg kg $^{-1}$ treated group respectively as compared to control.

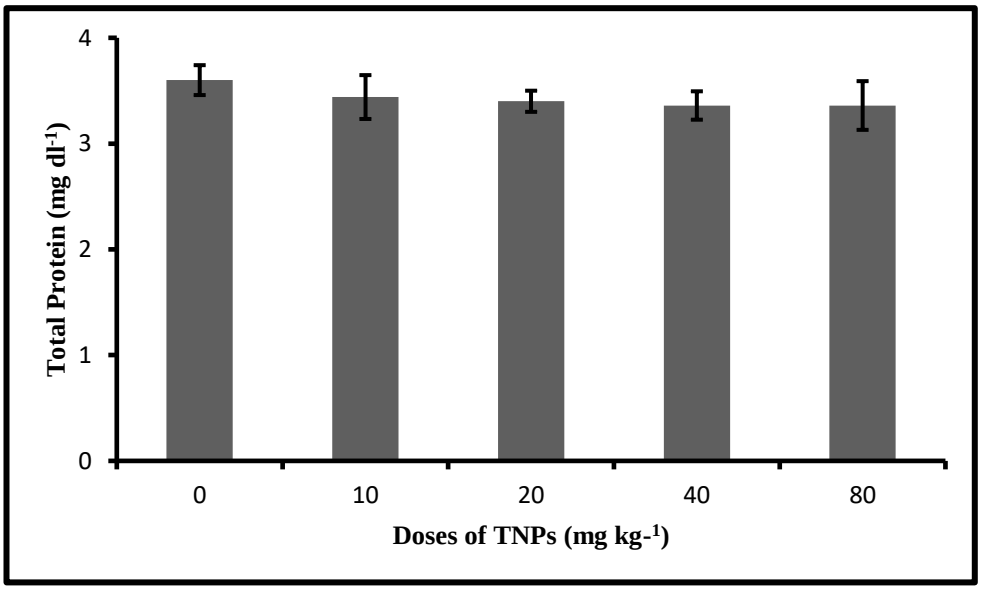


Fig. 13: Levels of Total Proteins in Chicken in Response to TNPs

This decrease may be due to high energy demand tempted by TNPs intoxication as suggested by Monfared and Soltani (2013), who reported significant decrease in the serum levels of total protein in rainbow trout when exposed to silver nanoparticles. Our results are also supported by a study conducted on rats in which no significant difference was found in total proteins at 0.5, 5, or 50 mg kg $^{-1}$

concentrations of TNPs through intra-tracheal instillation (Liu *et al.*, 2009; Liang *et al.,* 2009). While analyzing blood parameters in broiler at different ages it was discovered that the total protein level decreased at 21st day of their age, due to the high requirement of these proteins for growth (Silva *et al.*, 2008). As all proteins are synthesized by liver so the decrease in protein level might be an indicator of liver damage but not a definite and sensitive indicator because of the shorter half-lives of these proteins. A decrease in total protein at 50 mg kg $^{-1}$ and slight increase at 100 mg kg $^{-1}$ of TNPs was observed in rats after 14 days of exposure (Vasantharaja *et al.*, 2015). This difference may be due to difference in doses and exposure time.

4.4.2 Albumin

Albumin is a major group of proteins constituting more than half of the total proteins and blood sera. It is synthesized mainly in liver and prevents blood from leaking through vessels. It plays an important role in drug delivery through blood and healing of tissues.

Figure 14 represents trend by ALB levels with respect to dose of TNPs. Highest dose of 80 mg kg $^{-1}$ decreased by 12% as compared to control. This decrease may be due to high energy demand as in case of total protein (Monfared and Soltani, 2013).

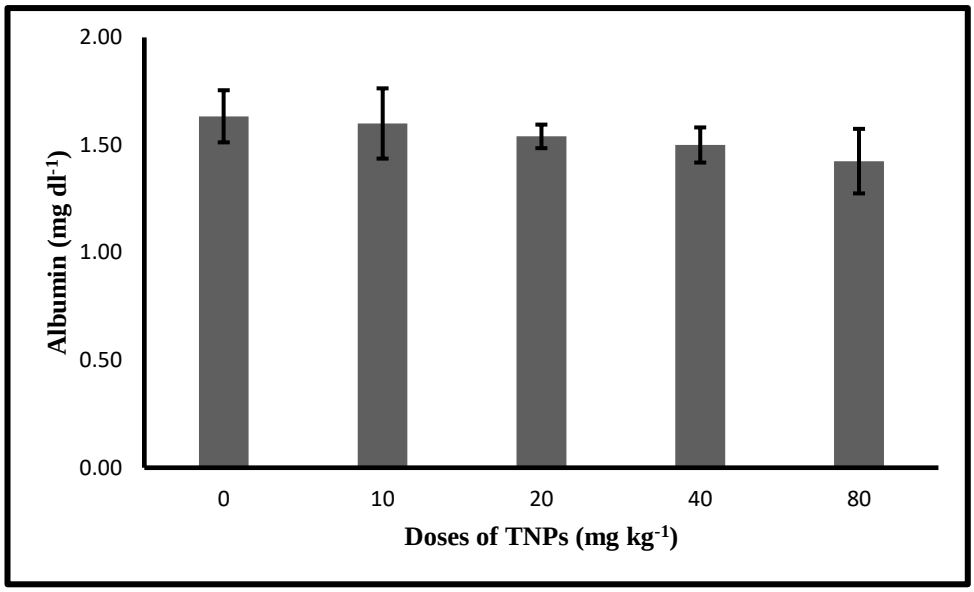


Fig. 14: Levels of Albumin in Chicken in Response to TNPs

Similar trends have been shown in other studies, an insignificant decrease in serum albumin was reported in rats when exposed to two groups of TNPs having specific surface area of 50 and 100 m^2 g^{-1} (Liang *et al.,* 2009). In another study 50 mg kg^{-1} of TNPs dose in rats through intra-tracheal instillation also reported no significant impact (Liu *et al.,* 2009). In another study, the mean values of serum albumin were within the normal range at concentration of 300 ppb of selenium nanoparticles in Wistar rats (Bunglavan *et al.,* 2014).

Contrary to our results, mice showed enhanced albumin levels at concentrations of 5, 10, 50, 100, and 150 mg kg^{-1} of Body Weight (BW) as compared to control group after 14 days exposure. Albumin level were significantly high at 50, 100 and 150 mg kg^{-1} treatments. This increased level was a sign of liver injury which was confirmed by histopathology (Ma *et al.,* 2009). This contrast may be due to difference in exposure routes and doses.

4.4.3 Cholesterol

As shown in the figure 15, cholesterol level decreased by 4% at 10, 1% at 20 mg kg^{-1} while it increased by 4 and 4.3% at 40 and 80 mg kg^{-1} respectively as compared to control group.

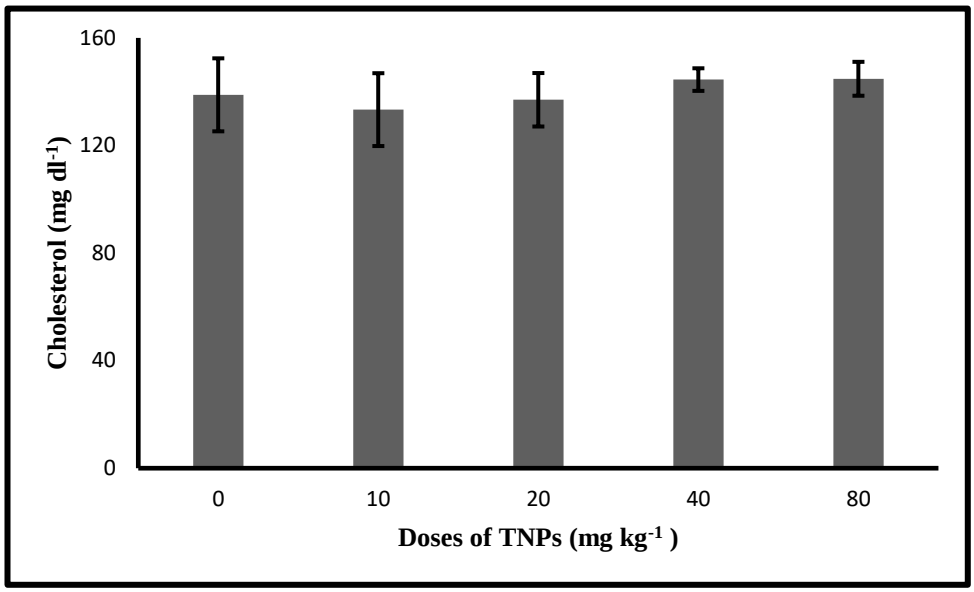


Fig. 15: Levels of Cholesterol in Chicken in Response to TNPs

Similar insignificant impact of TNPs has been reported on serum cholesterol of rats at 0, 2, 10, 50 mg kg^{-1} for 30 and 90 days after oral exposure (Chen *et al.*, 2015). Another study reported higher cholesterol levels ($p<0.05$) at 100 and 150 mg kg^{-1} of TNPs while lower dose of 5 mg kg^{-1} has 16 % lower cholesterol levels as compared to control group in mice (Ma *et al.*, 2009). Zinc oxide nanoparticles result an insignificant decrease in sera cholesterol of broiler chicken at 30, 60, 90 and 120 mg kg^{-1} treatment levels (Ahmadi *et al.*, 2013). Broilers feed with 2, 4 and 6 ppm of AgNPs kg^{-1} diet showed decrease in cholesterol levels as compared to control while high dose of 10 ppm AgNPs kg^{-1} diet had increased cholesterol level (Elkloub *et al.*, 2015).

A number of studies also reported significant increase in cholesterol levels, male Wistar rats showed an increase ($p<0.05$) in cholesterol levels at 50 and 100 mg

kg^{-1} of TNPs. Elevated cholesterol level is considered as a sign of liver toxicity because the bile duct hyperplasia found in male rats exposed to silver nanoparticles was accompanied with high cholesterol levels (Vasantharaja *et al.,* 2015). A significant difference was found in serum cholesterol levels of control and high dose (100 µg) of TNPs exposed mice through tracheal instillation (Chen *et al.,* 2013). Wistar rats showed significant decrease ($p<0.001$) in cholesterol level when exposed up to 300 ppb of selenium nanoparticles (Bunglavan *et al.,* 2014). In another study, significant decrease ($p<0.05$) in serum cholesterol at 10 mg kg^{-1} and significant increase ($p<0.05$) at 20 mg kg^{-1} were observed when rats were treated with nickel nanoparticles through intravenous injection. The increase at 20 mg kg^{-1} dose could be a sign of liver damage as increase in serum cholesterol has also been observed in some liver diseases (Magaye *et al.,* 2014).Type of nanoparticles, route of exposure, treatment time and experimental species are the major factors resulting in different impacts.

4.4.4 High Density Lipoprotein Cholesterol (HDLC)

HDLC is a form of cholesterol which helps the body to get rid of bad cholesterol by transporting cholesterol packets from blood to liver from where these are released from the body. High level of HDLC in blood serum reduces risk of heart diseases.

Figure 16 shows the relation of TNPs and HDLC levels in blood serum. 10 and 20 mg kg^{-1} treated groups have 98 mg dL^{-1} and 99 mg dL^{-1} of HDLC respectively while higher doses of 40 and 80 mg kg^{-1} groups have 100 mg dL^{-1} which is lower as compared to control group (103 mg dL^{-1}). TNPs had not significantly affected HDLC levels in treated and control groups.

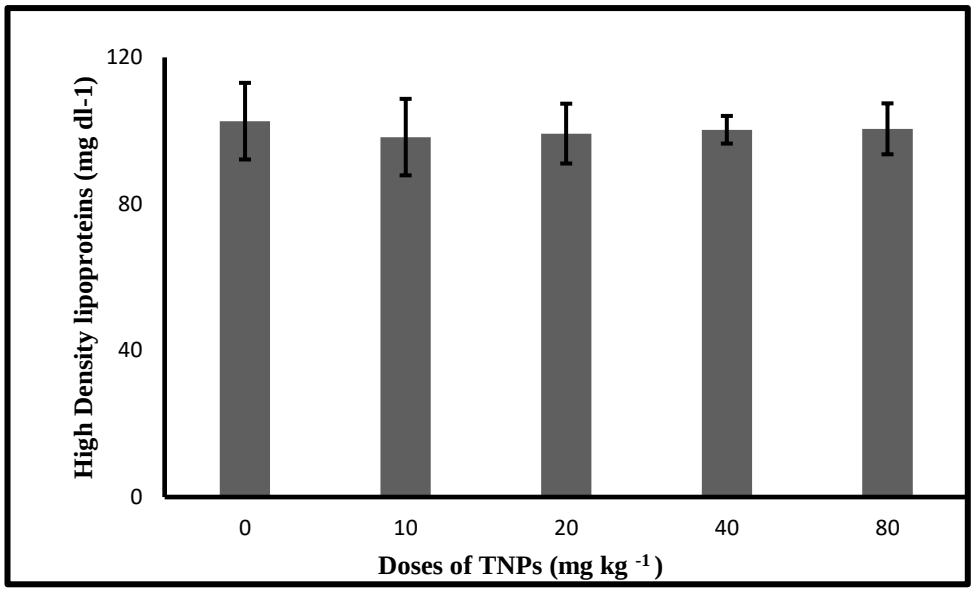


Fig. 16: Levels of HDLC in Chicken in Response to TNPs

Our findings are supported by a study on TNPs treated rats at 2, 10, 50 mg kg^{-1} BW doses and no significant change was observed in HDLC and cholesterol levels of control and treated groups (Chen *et al.,* 2015). In contrast to our results Chen et al., 2013 reported significant difference in HDLC and cholesterol levels of control and TNPs exposed mice. HDLC of 100 µg kg^{-1}per week treated mice was very low than control group which can cause a major disturbance in lipoproteins metabolism. Another study revealed significant increase in HDLC levels of Zinc oxide nanoparticles treated broilers (Ahmadi *et al.,* 2013). Studies have proved that there is a positive correlation between serum cholesterol and risk of atherosclerosis and HDLC is a protective agent against atherosclerosis in humans (Chen *et al.,* 2013).

4.4.5 Alanine aminotransferase (ALT)

ALT is mainly found in liver but its small amount is also found in muscles, kidneys, heart and pancreas. Normal serum levels of ALT is low but its level elevates in case of liver cells and membrane damage or death. Thus serum ALT level is used to diagnose liver health.

Figure 17 represents the ALT level in blood sera of chicks treated with selected TNPs doses. The figure shows that, with the increase in the concentration of TNPs, level of ALT in blood sera decreased insignificantly. In all experimental groups, ALT levels lowered by 10, 30, 40 and 46% respectively as compared to control group.

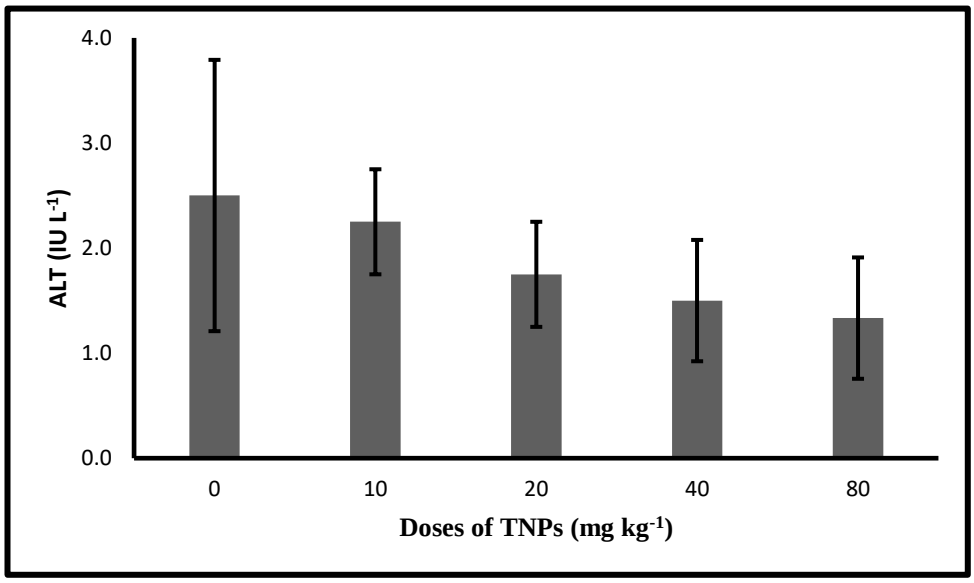


Fig. 17: Levels of ALT in Chicken in Response to TNPs

In the support of our results another study also reported no significant difference in ALT levels of control and exposure groups in rats at high dose of 50 mg kg^{-1} TNPs through intratracheal instillation. These results were also accompanied by no significant tissue damage (Liang *et al.,* 2009). Various doses (0.16, 0.4 and 1 g kg^{-1}) of TNPs induced no significant change in ALT levels of rats after 14 days of oral exposure (Bu *et al.,* 2010). No significant impact of TNPs on serum ALT was observed even at low dose (50 mg kg^{-1}) and high dose (100mg kg^{-1}) groups of rats (Vasantharaja *et al.*, 2015).

Similar decrease was reported in rats treated with silver nanoparticles of dose 100, 1000 and 5000 mg kg^{-1} daily for 7, 14 and 21 days alternately but contrary to our findings there was significant (P<0.001) reduction in the levels of ALT which was

dose and duration dependent (Adeyemi and Adewumi, 2014). This difference may be due to higher doses.

Contrary to our results, silver nanoparticles induced significant decrease ($p<0.05$) in ALT levels in treated broilers at doses of 20, 40, and 60 ppm Ag-NPs kg^{-1} diet for 42 days (Ahmadi, 2012). Another study reported significant increase in ALT levels in fish treated with concentrations of 300 and 1000 mg L^{-1} of silver nanoparticles. This increase is an indicator of liver damage (Monfared and Soltani, 2013).

Normally low ALT levels are considered as a sign of healthy liver but very low level also refers to low functional or non- functional liver, which cannot release ALT in blood serum. Patients of hepatitis C also have lower ALT levels in later stages. Lower levels of ALT can also be due to malnutrition or urinary tract infection.

4.4.6 Alkaline Phosphatase (ALP)

ALP is an enzyme found all over the body but in large amount in liver, pancreas, bones and bile duct. Any damage or destruction of these organ cells lead to an elevation of ALP in blood serum.

In the present study, maximum level of ALP was found in control group and it lowered insignificantly by 31, 28, 23 and 5% at 10, 20 40 and 80 mg kg^{-1} treated groups respectively. ALP at 80 mg kg^{-1} was 37% higher than 10 mg kg^{-1} group, which had lowest ALP level and then it increased continuously with increase in dose.

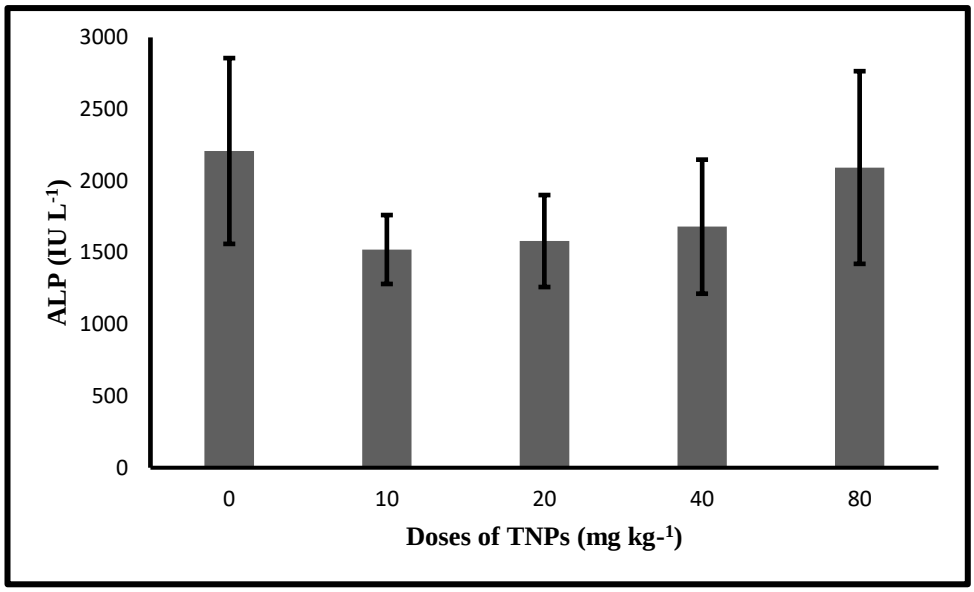


Fig. 18: Levels of ALP in Chicken in Response to TNPs

Our results are supported by TNPs treated rats at 50 and 100 mg kg^{-1} which showed 10 and 11% lower ALP levels respectively as compared to control (Vasantharaja *et al.*, 2015). A decrease in ALP levels of TNPs treated mice was observed at doses of 0, 324, 648, 972, 1296, 1944 and 2592 mg kg^{-1} for 14 days (Chen *et al.*, 2009).

A number of studies also reported contrary results of significant increase in ALP levels. TNPs at dose levels of 30, 50 and 70 mg kg^{-1} significantly ($p<0.01$) raised ALP levels in rats (Fatemeh and Mohammad, 2014). There was also significant increase ($p<0.05$) in ALP levels of mice at 100 and 150 mg kg^{-1} of TNPs (Ma *et al.*, 2009). TNPs @ 150 mg kg^{-1} were also reported to raise ALP and ALT levels in rats while liver damage was also confirmed by histological studies (Shakeel *et al.*, 2016). Silver nanoparticles also elevate ($p<0.05$) ALP level in broilers at doses of 20, 40, and 60 ppm Ag-NPs/kg diet for 42 days (Ahmadi, 2012). Silver nanoparticles were also reported to cause an increase ($p<0.05$) in ALP levels with the increasing length of treatment in rats (Adeyemi and Adewumi, 2014). Serum ALP levels also vary with

the development as mentioned by Silva *et al*., 2007 that ALP level in broiler chicks was higher at the age of 21 days due to enhanced bone development.

These fluctuations in ALP and ALT levels may be an indication of adaptive mechanisms by the chicken trying to offset stress induced by the exposure as mentioned by Adeyemi and Adewumi, 2014 as their dosages at 100 mg kg^{-1} of silver nanoparticles produced more significant alterations to the biochemical parameters than did the higher dosages at 1000 and 5000 mg kg^{-1} in rats.

4.4.7 Blood Urea Nitrogen (BUN)

Urea is a by-product of protein breakdown, produced in liver and excreted through urine. Level of BUN in blood serum shows health of kidneys and their functioning. When kidneys could not remove urea from body its level in blood increase. Heart failure, dehydration, or a diet high in protein can also lead to high BUN level. Whereas Liver disease, over hydration or damage or lower protein intake can also lower BUN level.

BUN level has shown significant decrease ($p<0.05$) in TNPs treated groups as compared to control as represented in the figure 19. BUN is 6 mg dL^{-1} at control while it decreased to 3.60 and 3.75 mg dL^{-1} in 10 and 80 mg kg^{-1} treated groups respectively.

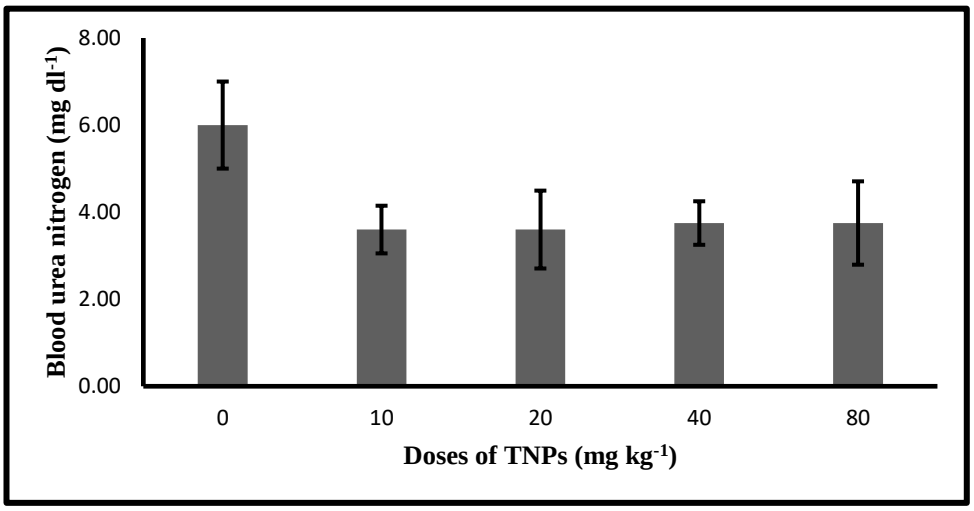


Fig. 19: Levels of BUN in Chicken in Response to TNPs

TNPs have not been reported to cause significant difference in BUN between control and TNPs treated mice and rats (Chen *et al.,* 2009: Bu *et al.,* 2010 and Liang *et al.,* 2009). 50 and 100 mg kg^{-1} of TNPs cause an increase in BUN in rats as compared to control group, which is an indication of kidney dysfunctioning (Vasantharaja *et al.,* 2015).

4.4.8 Creatinine (CR)

Creatinine is a chemical waste produced as a result of muscular activities. It is removed through kidneys. High levels of CR in blood is a sign of kidney dysfunction.

As the figure 20 represents that control and treated groups of chicken has same levels of CR in blood serum except for 80 mg kg^{-1} which has 12% elevated CR as compared to control and lower doses.

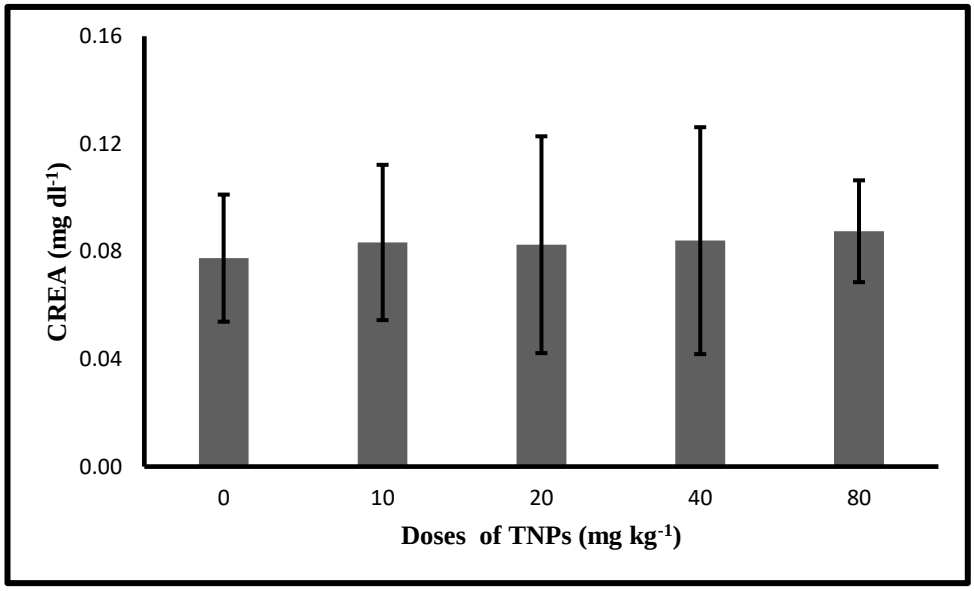


Fig. 20: Levels of CREA in Chicken in Response to TNPs

A number of studies confirmed this trend of CR as no changes in CR levels were observed at 0.16, 0.4, 1 g/kg, 50 and 100 mg kg^{-1} TNPs oral exposure of 14 days in rats (Bu *et al.,* 2010 and Vasantharaja *et al.,* 2015). Control and TNPs tracheal instilled treated rats at 0.5, 5, or 50 mg kg^{-1} doses also showed no difference in CR after 7 days (Liang *et al.,* 2009). Even 1, 10 and 20 mg kg^{-1} of nickel nanoparticles

induced no significant impact on CR and BUN of rats after 14 days exposure (Magaye *et al.*, 2014).

Intravenously injected TNPs (0, 140, 300, 645, or 1387 mg kg^{-1}) showed no significant impact on ALP, ALT, CREA and BUN of mice (Xu *et al.*, 2013). While silver nanoparticles at doses of 20, 40, and 60 ppm AgNPs kg^{-1} diet significantly affected ALT, ALP, CREA, TP, albumin, triglyceride, and cholesterol in broilers after 42 days of exposure, which may be due to oxidative stress which cause peroxidation of fats and release of free radicals (Ahmadi, 2012). Serum biochemical parameters even differ significantly in male and females of different indigenous ecotypes of broilers in Sudan (Elagib *et al.*, 2012).

4.4.9 Average Body Weight (BW) of Chicken

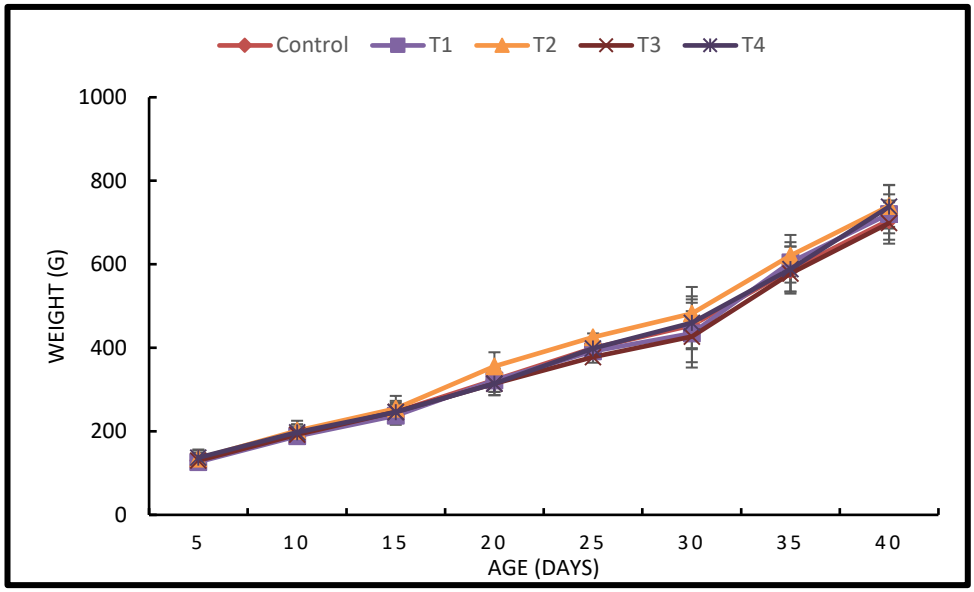


Fig. 21: Body weight of Local Chicken in Response to TNPs

The final BW of T4 is 32 g higher than control group while it is 17 g higher than T1. Among all groups, T2 had maximum BW which was 740 g after 40 days. There was no significant difference between BW of all treated and control groups (Figure 21). In our study, TNPs showed no significant impact on growth enhancement of native birds. Efficiency of Silver nanoparticles in growth parameters of chicken

embryo was investigated and results showed that silver nanoparticles significantly enhance muscle development without any adverse impacts (Sawosz *et al.,* 2012). Another study demonstrated that silver nanoparticles when provided in drinking water, they do not affect growth of chicken (Ahmadi and Kurdestany, 2010; Pineda *et al.,* 2012). *In ovo* injection of copper nanoparticles have been reported to have no effect on body weight of chicken (Pineda *et al.,* 2013).

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Application of TNPs had significantly affected plant growth in terms of root, shoot length biomass, chlorophyll content and grain yield. Maximum shoot length was observed at 700 mg kg^{-1} which was 20% improved as compared to control, while root length increased by 31% and root biomass by 10% at 600 mg kg^{-1}. Plants treated with 800 mg kg^{-1} showed maximum chlorophyll content. 700 mg kg^{-1} may be considered as optimum concentration level for maximum rice growth under given circumstances. No Ti was translocated to grains even at 1000 mg kg^{-1}. TNPs have also enhanced root hair development which was confirmed by SEM images, thus maximizing nutrients uptake and plant growth. TNPs were not associated with mortality and also played no significant role in development of native chicken. It is estimated from ALP and ALT results that TNPs did not induce any toxic impact but lower serum BUN levels can be an indication of disturbance in renal and liver functioning. These results also include diuretic effects which might be responsible for decreased toxicity of TNPs and lowering of BUN.

5.2 Future Perspectives

From the present study, significant effects on rice in response to TNPs have been observed. As Pakistan is an agricultural country and this sector has a major contribution in the national economy, this kind of studies could help to overcome food security issues by providing better crop yield. As a major segment of agriculture, poultry sector also needs to be adopted for enhanced production by improving immunity against pollutants and diseases. Along with positive impacts there are also

some limitations. Different factors such as experimental, environmental and climatic conditions especially temperature, humidity, sunlight, etc. affect the data sets. This work was done at small scale level in the laboratory at IESE, NUST. Pot experiment was performed in a local made greenhouse. Keeping in mind the different experimental conditions, these results may vary if experimentation tried in other parts of the country or regions across the globe with different environmental conditions. Similarly this work can also be tried in greenhouse with controlled conditions or growth chamber to better analyze the vegetative traits. This kind of methodology can be adopted for different plant species, using different sized nanoparticles, with different exposure time, at various growth stages and culture medium. Mode of application of nanoparticles can also be changed within the same and different plant species. Different results observed in case of different plant species, even if the same methodology was adopted. Experimentation on local chicken needs to be further extended and evaluated in different ways as biochemical blood profile is not sufficient to assess toxicity. Same experimentation can be done with smaller nanoparticles, accompanied with histopathological studies, with prolonged exposure time as toxic impacts start arising after longer exposure.

Apart from the potential benefits of this kind of studies, there are also some limitations that we could not ignore. At this stage, we could not claim with surety that this kind of technology is fully safe for human health and environment or it is harmful. Risks are associated with chronic exposure of humans to these nanoparticles, interaction with flora and fauna and their possible bioaccumulation effects have not been fully considered yet. Therefore, these concerns should be considered seriously before applying this study from laboratories to the field. The other limitations include the safe range of nanoparticles concentration, scalability of research and development

for prototype, industrial production and public's concern about health and safety issues. In this scenario, extensive research is necessarily required to resolve these concerns and provide conclusive information.

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