RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume Issue : ()

Synthesis, Characterization and Safety Assessment of Zinc, Manganese and Copper Nanoparticles for Incorporation in Broiler Chicken Feed

T.B. Gowtham[1], J. Ramesh[1], R. Karunakaran[2], N. Karthikeyan[3], P. Raja[4]

10.18805/ajdfr.DR-1876

ABSTRACT

Background: Zinc, manganese and copper are important trace minerals in broiler diet. Traditionally, these elements when generally supplemented as inorganic salts, their bioavailability is poor and resulted in mineral antagonism. To overcome these issues, such minerals were supplemented as Nanoparticles due to their higher bioavailability and efficacy. Hence, the current study was aimed to synthesize, characterize and evaluate the cytotoxic effect of nano forms of zinc, manganese and copper.

Methods: Nano forms of zinc, manganese and copper were synthesized through physical method using planetary ball mill. In this study particle size, shape, zeta potential and elemental concentration were determined by using particle size analyser (PSA), transmission eectron microscope (TEM) and inductively coupled plasma mass spectrometry (ICP-MS). The toxicity was analysed by MTT assay against *vero* cell line.

Result: The results revealed that the zinc, manganese and copper nanoparticles synthesized using planetary ball mill were nano in size (less than 100 nm) and pure in nature. Further, these nanoparticles are found to be safe feed supplements and their cell inhibition rate is dose dependent in MTT assay.

Key words: Cell cytotoxicity, Manganese and copper, Nano forms of zinc, Particle size, Safety, Zeta potential.

INTRODUCTION

Zinc, manganese and copper are important trace minerals in poultry nutrition. In birds, zinc is necessary for proper feathering, growth, skeletal development, skin quality, reproduction and disease resistance through boosting the immune system (Naz *et al*. 2016). Similarly, manganese is required for embryonic development, weight gain, bone growth, blood sugar homeostasis, immunological function, cellular energy, blood coagulation, cellular defense against free radicals and reproduction (Olgun, 2017). Copper is also regarded as a critical trace element in poultry diet and it aids in the creation of connective tissues like collagen and elastin, as well as the development of the nervous system through dopamine synthesis (Mroczek-Sosnowska *et al*., 2013).

Traditionally, trace minerals have been supplemented in poultry diets using inorganic salts such as zinc oxide, manganese oxide or sulphate and copper sulphate. However, in recent years there are increased concerns about the usage of inorganic trace mineral salts due to their poor bioavailability and potential to cause mineral antagonism, which impairs absorption of minerals and increases environment pollution.Minerals, particularly trace minerals, have a limited bioavailability in the bodies of animals (Star *et al*., 2012). Trace element nanoparticles can minimise mineral antagonism in the gut, resulting in the increased absorption and less excretion in the environment.

Further, these may also help to improve the immunological responses and digestive efficiency of birds, resulting in increased feed efficiency (Marappan *et al*. 2017).

[1]Department of Animal Nutrition, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.
[2]Dean, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.
[3]Department of Poultry Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.
[4]Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.

Corresponding Author: J. Ramesh, Department of Animal Nutrition, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamil Nadu, India.
Email: rameshnutrition@gmail.com

How to cite this article: Gowtham, T.B., Ramesh, J., Karunakaran, R., Karthikeyan, N. and Raja, P. (2022). Synthesis, Characterization and Safety Assessment of Zinc, Manganese and Copper Nanoparticles for Incorporation in Broiler Chicken Feed. Asian Journal of Dairy and Food Research. DOI: 10.18805/ajdfr.DR-1876.

Submitted: 21-01-2022 **Accepted:** 03-08-2022 **Online:** 18-08-2022

Nanoparticles can be produced through different techniques, which can be broadly classified into top-down method and bottom-up method. Top-down method is also called as physical method whereas; bottom-up method is also called as chemical method. Top-down method involves breaking down of a bulk material into nano-sized particles using cutting, grinding and etching techniques, *i.e.,* nano materials are produced from larger entities without atomic-level control (Umer *et al.* 2012). Ball milling is the simplest mechanical top down (destructive) method of synthesis of nanoparticles.

Nanomaterial toxicity is strongly influenced by factors such as particle size and surface area (Nel *et al.*, 2009). Because each type of nanoparticle has its own unique physicochemical properties, the harmful effects on cells may differ. Some harmful effects are irreversible and permanent, resulting in cell death, whereas others are reversible if nanoparticle exposure is eliminated, allowing cells to proliferate normally. Hence, this study was carried out to synthesize, characterize and evaluate the cytotoxicity effect of nano forms of zinc, manganese and copper.

MATERIALS AND METHODS

Synthesis of nano forms of zinc, manganese and copper

Nano forms of zinc, manganese and copper were synthesized through physical method at the Department of Animal Nutrition, Madras Veterinary College, Chennai during 2021. Ball milling was the technique employed for the synthesis. The procedure of synthesis of nano forms of zinc, manganese and copper was optimized by method development. Nano form of zinc was synthesized by grinding feed grade zinc oxide for eight hours in planetary ball mill (Retsch PM 100) @ 250 cycles/min using fifty numbers of zirconium balls with 5 mm diameter in 50 ml capacity zirconium jar.

Nano form of manganese was synthesized by grinding feed grade manganese sulphate for four hours in planetary ball mill (Retsch PM 100) @ 250 cycles/min using fifty numbers of zirconium balls with 5 mm diameter in 50 ml capacity zirconium jar.

Nano form of copper was synthesized by grinding feed grade copper sulphate for two hours in planetary ball mill (Retsch PM 100) @ 250 cycles/min using fifty numbers of zirconium balls with 5 mm diameter in 50 ml capacity zirconium jar.

Characterization of nano forms of zinc, manganese and copper

The properties of the materials are likely to be far different from the bulk materials, when the particle size is reduced to nano size. In the present study, Transmission Electron Microscopy, Particle Size Analyzer, X Ray Diffractometer, Fourier Transform Infra-red (FTIR) spectroscopy and Inductively Coupled Plasma Mass Spectrometry were used to analyze the properties of synthesized nanoparticles like particle size, shape, zeta potential and elemental concentration. The nanoparticle yield was calculated by weighing the final product and comparing it to the precursor used.

In vitro cytotoxicity assay

In vitro cytotoxicity assay was carried out in *vero* cell line (African green monkey kidney cell line) to ensure the safety of nano particle source of zinc, manganese and copper as per the method of Mosmann, (1983). Serially diluted nano particles were incubated in ninety-six well plates for 72 hours containing *vero* cell line at the concentration of 10^6 cells/ml with solvent following standard protocol. The concentrations of nano zinc selected were 20, 40, 60, 80, 100, 250, 500, 800 µg/ml. Nano manganese was added at the concentrations of 25, 50, 75, 100, 250, 500, 750, 1000 µg/ml.Similarly, nanocopper was added at the concentrations of 5, 10, 15, 20, 25, 50, 100, 150 µg/ml. The level of concentrations for each nano form of mineral was fixed considering their standard recommended incorporation level (BIS, 2007) in poultry feed. The samples were run in triplicates.

At the end of incubation, colorimetric method measured the reduction of yellow 3- (4, 5 dimethythiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase of live cell. Mean was calculated from the triplicate readings of OD values for each sample. The absorbance value of blank was subtracted from all the samples in order to derive corrected absorbance values. From the corrected absorbance values, calculation was done using the following formula:

$$\text{Per cent viable cells} = \frac{\text{Corrected absorbance sample}}{\text{Corrected absorbance control}} \times 100$$

RESULTS AND DISCUSSION

Synthesis of nano forms of zinc, manganese and copper

Nano particle source of zinc, manganese and copper were successfully synthesized through physical method using planetary ball mill. Zinc oxide, manganese sulphate and copper sulphate were used as sources to produce zinc, manganese and copper nanoparticles respectively. Optimum conditions (such as grinding jar size, sample volume, size and number of grinding balls used, speed of rotation in rpm and duration of grinding) required for preparation of nano forms of zinc, manganese and copper were standardized. The major advantages of mechanical milling are simpler to operate, low cost of production of nanoparticles and the possibility to produce it in large scale (McCormick and Froes, 1998).

Characterization of nano forms of zinc, manganese and copper

The product yield, particle size, shape, zeta potential and elemental concentration of the prepared nano particles are presented in Table 1. The recovery percentage of nano particle source of zinc, manganese and copper synthesized through ball milling method were 95.75, 95.50 and 96.25 respectively.

The particle size assessed through both particle size analyser and Transmission Electron Microscopy confirmed that the synthesized nano forms of zinc, manganese and copper were less than 100 nm. The TEM image of the synthesized nano forms of zinc, manganese and copper are presented in Fig 1, Fig 2 and Fig 3 respectively. Mean size assessed through TEM were 80.64 nm, 20.33 nm and 12.98 nm for synthesized nano forms of zinc, manganese and copper respectively. Whereas, mean size assessed through particle size analyser were 18.20 nm, 16.90 nm and 70.90 nm for synthesized nano zinc, manganese and copper respectively.

The X-Ray diffraction (XRD) pattern of synthesized nano particle source of zinc, manganese and copper are presented in Fig 4, Fig 5 and Fig 6 respectively. The data recorded were analyzed using Jade 6.0 software. X-Ray diffraction pattern confirms that the synthesized nano particle source of zinc, manganese and copper were free of impurities as it does not contain any characteristic XRD peaks other than their respective element peak and the samples are nano in nature.

FTIR spectroscopy is used to estimate the type of the functional groups and their involvement during bio reduction.

Table 1: Product yield (recovery %), particle size, zeta potential and elemental concentration in nano forms of zinc, manganese and copper (Mean# ± SE).

Chemical name of the source	Characterization parameters		
	Zinc oxide	Manganese sulphate	Copper sulphate
Recovery (%)	95.75 ± 1.05	95.50 ± 0.75	96.25 ± 1.25
Size (assessed through TEM) (nm)	80.64 ± 6.45	20.33 ± 5.68	12.98 ± 4.73
Size (assessed through particle size analyser) (nm)	18.20 ± 5.21	16.90 ± 4.58	70.90 ± 3.24
Zeta potential (mV)	-33.50 ± 3.54	-29.90 ± 2.56	-25.10 ± 2.49
Elemental concentration (%)	76.57	34.04	21.52
Shape	Hexagonal	Spherical	Spherical

#Mean of six observations.

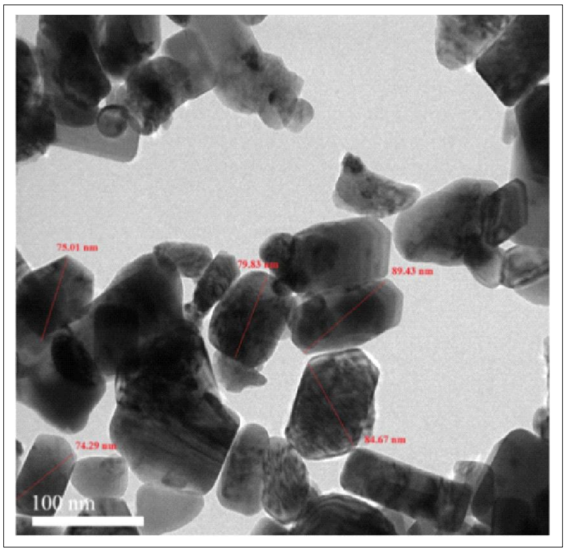

Fig 1: Transmission electron microscope image of synthesized nano zinc with average particle size of 80.64 nm and hexagonal in shape.

The FTIR spectrum of synthesized nano particle source of zinc revealed well-defined peaks at around 552 cm^{-1} and 1163 cm^{-1}. The FTIR spectrum of synthesized nano particle source of mangane serevealed well-defined peaks at around 625 cm^{-1} and 3137 cm^{-1}. The FTIR spectrum of synthesized nano particle source of copperr evealed well-defined peaks at around 565 cm^{-1} and 3104 cm^{-1}.In the present study, the unique finger printing for zinc, manganese and copper were evident in the respective nano particle source of zinc, manganese and copper respectively.The observed FTIR results confirmed that synthesized zinc, manganese and copper nanoparticles were without any significant impurities.

The elemental concentrations were analyzed by using Inductively Coupled Plasma Mass Spectrometry (ICPMS). The results revealed that nano zinc contains 76.57% elemental zinc, nano manganese contains 34.04% elemental manganese and nano copper contains 21.52% copper. The elemental concentration of the synthesized

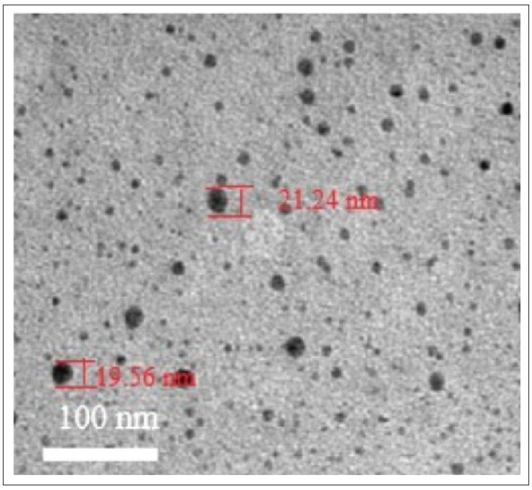

Fig 2: Transmission electron microscope image of synthesized nano manganese with average particle size of 20.33 nm and spherical in shape.

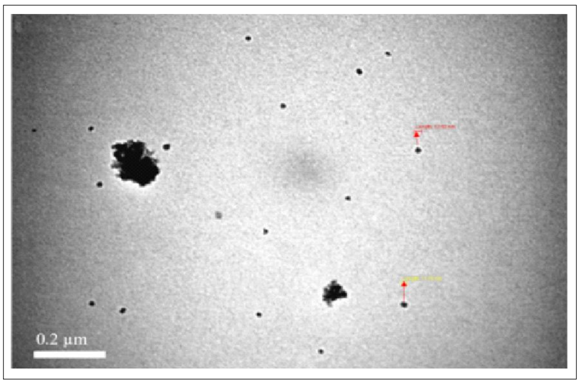

Fig 3: Transmission electron microscope image of synthesized nano copper with average particle size of 12.98 nm and spherical in shape.

nano particle sources of zinc, manganese and copper were almost same as that of the original mega particle source from which they were produced. This indicates no loss in zinc, manganese and copper as a result of ball milling.

The shape of the synthesized nanoparticles were analysed through TEM. The shapes of nano manganese and copper obtained in this study were spherical. Spherical shape of nano particles has an advantage of not causing injury while being transported across the tissue (Xu *et al*. 2012). Thus nano particle source of manganese and copper produced will not cause damage to cells / tissues on oral administration. Though the shape of nano zinc synthesized was hexagonal, it was reported that hexagonal shaped zinc oxide nanoparticles are safe to administer orally in poultry (Mohd Yusof *et al*. 2021).

Nanoparticles with zeta potential values more than +25 mV or less than -25 mV are known to be extremely stable. Due to Van Der Waal inter-particle interactions, dispersions with a low zeta potential value will eventually coalesce (Nanocomposix, 2012). In our study it was

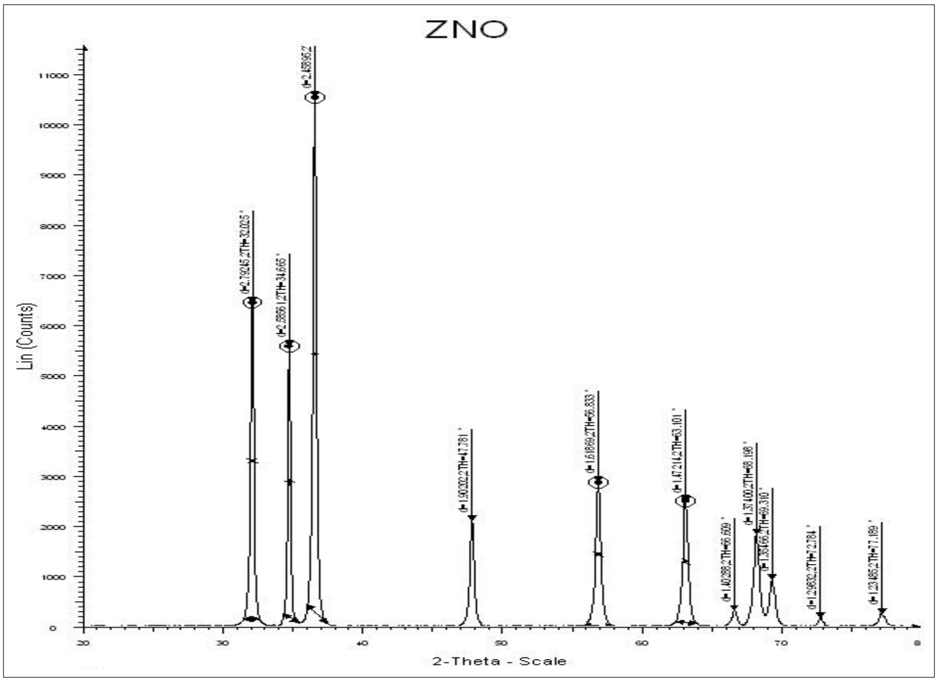

Fig 4: X ray diffraction pattern of nano zinc.

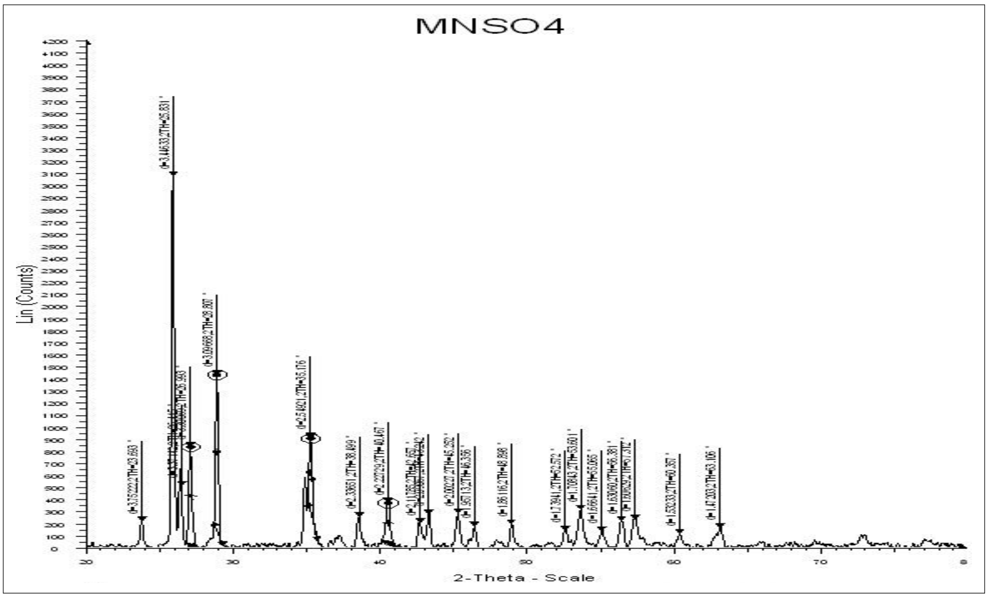

Fig 5: X ray diffraction pattern of nano manganese.

observed that the zeta potential (mV) of the synthesized zinc, manganese and copper nanoparticles were -33.50, -29.90 and -25.10 respectively. Thus the zinc, manganese and copper nanoparticles synthesized in our study could be of good stability.

Concurring with this study, nano particles of zinc, manganese and copper having similar size and shape were produced by other researchers. Geetha *et al*. (2016) revealed that the TEM images of ZnO nanoparticles confirmed that the particles were hexagonal in shape. The average particle size was found to be 50-100 nm. Cherian *et al*. (2016) reported that the SEM images of manganese nanoparticles were found to be spherical in shape with the diameter range of 40.5 to 70 nm. In FTIR a strong absorption peak was found at 625 cm^{-1} which is similar to our result. Sufeesh *et al*. (2019) synthesized copper nanoparticles through physical method using ball mill. The synthesized copper nanoparticles were spherical in shape with the average particle size of 14.67 nm and zeta potential of -12.3 mV. The elemental concentration of copper in the synthesized nanoparticle was found to be 23.26%.

In vitro cytotoxicity assay

The results of MTT assay on per cent cell viability for synthesized nano forms of zinc, manganese and copper are presented in Table 2. A value above 75 per cent indicates good cell viability. Nano-zinc showed good cell viability up to the concentration 250 µg/ml. The per cent cell viability for nano zinc at 250 µg/ml concentration was 75.57%. This safe concentration level of 250 µg/ml is equivalent to 250 mg/ kg which is more than 3 folds of recommended dose of 80 ppm zinc in broiler chicken diet.

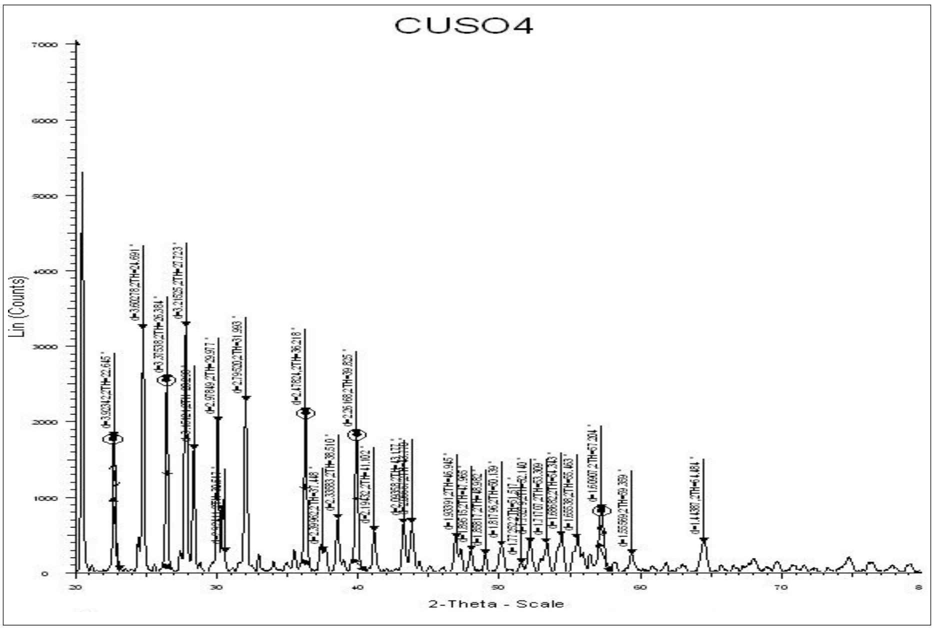

Fig 6: X ray diffraction pattern of nano copper.

Table 2: Effect of nano forms of zinc, manganese and copper on per cent cell viability ofAfrican Green Monkey Kidney (*VERO*) cell line determined by MTT assay (Mean#±S.E.).

Nano zinc		Nano manganese		Nano copper	
Concentration (µg/ml)	% cell viability	Concentration (µg/ml)	% cell viability	Concentration (µg/ml)	% cell viability
20	96.88±0.56	25	97.29±0.22	5	95.77±0.64
40	93.19±0.73	50	94.39±1.21	10	91.22±1.25
60	87.22±1.12	75	89.02±0.86	15	84.82±1.66
80	80.48±1.08	100	82.47±0.46	20	83.48±0.14
100	79.25±0.25	250	78.37±0.45	25	78.19±0.06
250	75.57±0.26	500	75.85±1.08	50	76.24±0.48
500	54.82±1.29	750	60.40±0.11	100	60.70±0.54
800	40.14±0.88	1000	37.32±0.91	150	48.03±0.75

#Mean of three independent experiments.

Nano-manganese showed good cell viability up to the concentration 500 µg/ml. The per cent cell viability for nano-manganese at 500 µg/ml concentration was 75.85%. This safe concentration level of 500 µg/ml is equivalent to 500 mg/kg which is 5 folds of recommended dose of 100 ppm manganese in broiler chicken diet. Nano-copper showed good cell viability up to the concentration 50 µg/ml. The per cent cell viability for nano-copper at 50 µg/ ml concentration was 76.24%. This safe concentration level of 50 µg/ml is equivalent to 50 mg/ kg which is more than 4 folds of recommended dose of 12 ppm copper in broiler chicken diet.

Inorganic minerals are relatively inert in their bulk form. However, cytotoxicity assay of any nano particle source is essential, as the particle size decreases, toxicity increases. Nano particles interact with proteins and enzymes within cells and they can interfere with the antioxidant defense mechanism leading to formation of reactive oxygen species, which further causes inflammatory response and destruction of the mitochondria finally leading to apoptosis or necrosis (Scharand *et al*. 2010).

In a cytotoxicity study, Saranya*et al*. (2017) studied the *in vitro* cytotoxic effects of green synthesized ZnO nanoparticles in Vero, PK 15 and MDBK cell lines. The ZnO nanoparticles revealed better cell viability at lower concentration (10 µg/100 ìl) in all type of cells (Vero, PK 15 and MDBK cells). Similar results were reported by Tabassum *et al*. (2021) who observed the in vitro cytotoxic effects of manganese nanoparticles in human breast cancer cell line. The results showed that there is significant dose- dependent inhibitory effect of manganese nanoparticles. When the concentration of manganese nanoparticles increased from 25 to 125 µg/ml, the cell viability decreased from 94% to 61 %. Sufeesh *et al*. (2019) studied the *in-vitro* cytotoxic effects of copper nanoparticles in vero cell lines. The results of the study revealed that copper nanoparticles showed good cell viability at 100 µg/ ml concentration. Hence, in this study, the results obtained revealed that safest inclusion level of nano zinc, manganese and copper in broiler chicken feed is up to 250 ppm, 500 ppm and 50 ppm respectively.

CONCLUSION

Zinc, manganese and copper nano particles would be successfully and effectively synthesized through physical method using planetary ball mill and the same could be characterized by several techniques as followed in this study for its quantity, size, shape, stability and purity.

The result of the present study indicated that nano forms of zinc manganese and copper had all the characteristics of nano particle. Based on the result of *in vitro* cytotoxicity assay we would say that nano forms of zinc, manganese and copper are non-toxic at our inclusion level. Hence, the synthesized zinc, manganese and copper nanoparticles could be used as a feed supplement for broiler chicken at the dosage of 80 ppm, 100 ppm and 12 ppm respectively as per the BIS specifications.

ACKNOWLEDGEMENT

The authors are very much grateful to the authorities of Tamil Nadu Veterinary and Animal Sciences University Chennai for providing necessary facilities to carry out this research study.

Conflict of interest: None.

REFERENCES

BIS, (Bureau of Indian Standards). (2007). Indian Standard Poultry Feeds - Specification. 5th Edn. New Delhi.

Cherian, E., Rajan, A. and Baskar, G. (2016). Synthesis of manganese dioxide nanoparticles using co-precipitation method and its antimicrobial activity. International Journal of Modern Science and Technology. 1(1): 17-22.

Geetha, M.S., Nagabhushana, H. and Shivananjaiah, H.N. (2016). Green mediated synthesis and characterization of ZnO nanoparticles using Euphorbia Jatropa latex as reducing agent. Journal of Science Advanced Materials and Devices. 1(3): 301-310.

Marappan, G., Beulah, P., Kumar, R.D., Muthuvel, S. and Govindasamy, P. (2017). Role of nanoparticles in animal and poultry nutrition: Modes of action and applications in formulating feed additives and food processing. International Journal of Pharmacology. 13(7): 724-731.

McCormick, P.G. and Froes, F.H. (1998).The fundamentals of mechanochemical processing. The Journal of the Minerals, Metals and Materials Society. 50(11): 61-65.

Mohd Yusof, H., Rahman, A., Mohamad, R., Hasanah Zaidan, U. and Samsudin, A.A. (2021). Antibacterial potential of biosynthesized zinc oxide nanoparticles against poultry-associated foodborne pathogens: An *in vitro* study. Animals. 11(7): 2093.

Mosmann, T. (1983). Rapid calorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 65: 55-63.

Mroczek-Sosnowska, N., Batorska, M., Lukasiewicz, M., Wnuk, A., Sawosz-Chwaliboìg, E., Jaworski, S. and Niemiec, J.A.N. (2013). Effect of nanoparticles of copper and copper sulfate administered in ovo on hematological and biochemical blood markers of broiler chickens. Annals of Warsaw University of Life Sciences-SGGW. Animal Science. 52.

Nanocomposix. (2012). Zeta Potential Analysis of Nanoparticles. Nanocomposix. 1(1): 1-6.

Naz, S., Idris, M., Khalique, M.A., Alhidary, I.A., Abdelrahman, M.M., Khan, R.U., Chand, N., Farooq, U. and Ahmad, S. (2016). The activity and use of zinc in poultry diets. World's Poultry Science Journal. 72(1): 159-167.

Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M., Somasundaran, P. and Thompson, M. (2009). Understanding biophysicochemical interactions at the nano-bio interface. Nature materials. 8(7): 543-557.

Olgun, O. (2017). Manganese in poultry nutrition and its effect on performance and eggshell quality. World's Poultry Science Journal. 73(1): 45-56.

Saranya, S., Vijayaranai, K., Pavithra, S., Raihana, N. andKumanan, K. (2017). *In vitro* cytotoxicity of zinc oxide, iron oxide and copper nanopowders prepared by green synthesis. Toxicology Reports. 4: 427-430.

Schrand, A.M., Rahman, M.F., Hussain, S.M., Schlager, J.J., Smith, D.A. and Syed, A.F. (2010). Metal based nanoparticles and their toxicity assessment. Wiley interdisciplinary reviews: Nanomedicine and Nanobiotechnology. 2(5): 544-568.

Star, L., Van der Klis, J.D., Rapp, C. and Ward, T.L. (2012). Bioavailability of organic and inorganic zinc sources in male broilers. Poultry Sci. 91(12): 3115-3120.

Sufeesh, S.G., Ramesh, J., Karunakaran, R., Vijayarani, K. and Parthiban, M. (2019). Safety level of nano copper and nano cobalt in dairy cattle feed. Indian Veterinary Journal. 96(1): 46-47.

Tabassum. N., Chaturvedi, V.K., Yadav, C.B., Singh, V. and Singh, M.P. (2021). *In vitro* cytotoxicity and antioxidant efficiency of synthesized mixed phase manganese oxide nanomaterial. J. Exp. Zool. India, 24: 95-100.

Umer, A., Naveed, S. and Ramayan, N. (2012). Selection of a suitable method for the synthesis of copper nano particles. Nano Brief Reports and Reviews. 7(5): 12-18.

Xu, M., Li, J., Iwai, H., Mei, Q., Fujita, D., Su, H., Chen, H. and Hanagata, N. (2012). Formation of nano-bio-complex as nanomaterials dispersed in a biological solution for understanding nanobiological interactions. Scientific Reports. 2(1): 1-6.