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Original Article

Effects of copper nanoparticles on performance, muscle and bone characteristics and serum metabolites in broilers

Efeitos de nanopartículas de cobre no desempenho, características musculares e ósseas e metabólitos séricos em frangos de corte

S. S. Abdullah[a], S. Masood[a*], H. Zaneb[a], I. Rabbani[b], J. Akbar[c], Z. H. Kuthu[c], A. Masood[d] and E. Vargas-Bello-Pérez[e*]

[a]University of Veterinary and Animal Sciences, Department of Anatomy and Histology, Lahore, Pakistan
[b]University of Veterinary and Animal Sciences, Department of Physiology, Lahore, Pakistan
[c]University of Poonch, Faculty of Veterinary and Animal Sciences, Rawalakot, Azad Jammu and Kashmir, Pakistan
[d]Islamia University, Department of Anatomy and Histology, Bahawalpur, Pakistan
[e]University of Copenhagen, Faculty of Health and Medical Sciences, Department of Veterinary and Animal Sciences, Frederiksberg, Denmark

Abstract

Three hundred and twenty day old Hubbard broilers were randomly allocated to four treatments (8 replicates, 10 birds/pen) and were raised under standard management conditions. Birds in the first group served as control and were fed a corn based diet, while birds in the remaining three groups i.e.; A, B and C were fed with a basal diet supplemented with copper nanoparticles (CuNP) at 5, 10 and 15 mg /kg of diet respectively for 35 days. Supplementation of CuNP linearly increased (P≤0.05) body weight (BW), average daily weight gain (ADWG) and feed intake (FI) in broilers. Uric acid, glucose levels in blood and feed conversion ratio (FCR) reduced linearly (P≤0.05) with CuNP supplementation in diet. Supplementation of CuNP in the diet also linearly increased (P≤0.05) tibia weight, length, diameter, weight/length index (W/L) and Tibiotarsal index (TT index). Inclusion of CuNP in broilers diet linearly increased the measured parameters of muscle i.e.; pH, fiber diameter, fiber cross-sectional area, fascicle diameter, fascicle cross-sectional area (P≤0.05). Concentration of copper, iron, calcium and phosphorous in blood also increased line-arly (P ≤ 0.05) with CuNP supplementation. Overall, CuNP positively affected the growth performance, histological characteristics of muscles, bone strength and serum metabolites in broilers.

Keywords: fascicle, fibre, glucose, robusticity index, tibia, uric acid.

Resumo

Frangos de corte Hubbard com 320 dias de idade foram alocados aleatoriamente em quatro tratamentos (8 repetições, 10 aves/curral) e foram criados em condições de manejo padrão. As aves do primeiro grupo serviram como controle e foram alimentadas com uma dieta à base de milho, enquanto as aves dos três grupos restantes, ou seja, A, B e C, foram alimentadas com dieta basal suplementada com nanopartículas de cobre (CuNP) a 5, 10 e 15 mg/kg de dieta, respectivamente, por 35 dias. A suplementação de CuNP aumentou linearmente (P ≤ 0,05) o peso corporal (PC), o ganho de peso médio diário (GPDA) e o consumo de ração (FI) em frangos de corte. O ácido úrico, os níveis de glicose no sangue e a conversão alimentar (TCF) reduziram linearmente (P ≤ 0,05) com a suplementação de CuNP na dieta. A suplementação de CuNP na dieta também aumentou linearmente (P ≤ 0,05) peso, comprimento, diâmetro, índice peso/comprimento (P/L) e índice tibiotársico (índice TT) da tíbia. A inclusão de CuNP na dieta de frangos de corte aumentou linearmente os parâmetros medidos de músculo, ou seja; pH, diâmetro da fibra, área da seção transversal da fibra, diâmetro do fascículo, área da seção transversal do fascículo (P ≤ 0,05). A concentração de cobre, ferro, cálcio e fósforo no sangue também aumentou linearmente (P ≤ 0,05) com a suplementação de CuNP. No geral, CuNP afetou positivamente o desempenho de crescimento, características histológicas dos músculos, resistência óssea e metabólitos séricos em frangos de corte.

Palavras-chave: fascículo, fibra, glicose, índice de robustez, tíbia, ácido úrico.

Introduction

The skeleton system provides shape to the body, protects internal organs, helps in locomotion and serves as storage of several minerals (Toba et al., 2000). Bone weakness is one of the major issue, which causes economic losses due to mortality and disapproval within processing plant (Rath et al., 2000). Weaker skeleton and bones result in

*e-mail: evargasb@sund.ku.dk; saima.masood@uvas.edu.pk
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low feed intake of feed due to slow motility and it results in lower weight gain and reduced egg production. Long bones such as tibia, femur and metatarsus are the key factors of meat quality (Orban et al., 1999). The risk of bone fracture is increased with low bone mineralization (Blake and Fogelman, 2002).

Muscle fibers build up the total mass of the muscle. Fibre size, fibre cross-sectional area and total number of fibers are important characteristic for meat quality (Rehfeldt and Kuhn, 2006). Meat tenderness correlates with muscle fiber thickness and meat quality is linked with the thickness of muscle fibers (Genchev et al., 2008).

Copper is an important trace element in the body and plays an important role in bone development (Shen et al., 2022). Copper is required for the normal functioning of lysyl oxidase enzyme, which plays a crucial role in the deposition of calcium and phosphorus in bones. A copper deficient diet results in the decreased bone strength and results in fractures (Gau et al., 2021). This phenomenon is explained by the decreased activity of osteoblasts whereas the activity of osteoclasts remains normal (Shen et al., 2022). Therefore, the process of bone formation requires proper supply of copper throughout the life (Palacios, 2006)

Copper is important for cross linking of collagen and elastin fibers and it is important for bones development (Miggiano and Gagliardi, 2005). Collagen is involved in the development of the bone, cartilage and muscles. In several domesticated species, meat toughness also occurs as a result of collagen accumulation (Bogucka et al., 2018).

Trace minerals such copper, zinc and manganese are important for the animal's metabolic activities (Soetan et al., 2010). Copper is used as a growth promoter and an antimicrobial agent in poultry. Currently copper sulphate is being used as the main source of copper. This inorganic form of copper has reduced bioavailability and a major portion of it is eliminated from the body which cause pollution and economic losses (Scott et al., 2018a). Copper from dissimilar sources [i.e., oxide, chloride, sulphate and tribasic copper] have been used in poultry feed to assess its bioavailability (Pang et al., 2009). Studies have revealed that the nano form of copper is greatly bioavailable because of the small size and an increased surface area. Because of a smaller size, Copper nanoparticles (CuNP) can easily pass the gasttointestinal tract (GIT) mucosa, taken up by the villus epithelium and are easily absorbed in blood (Scott et al., 2018a).

Mroczek-Sosnowska et al. (2015) reported that CuNP can increase growth performance in broiler compared to copper sulphate and resulted in an increased muscle development during embryonic life as a result of *in ovo* administration of nanoparticles.

Physical characteristics of bone were also improved by *in ovo* supplementation of CuNP by the stimulation of proliferating cell nuclear antigen [PCNA]-positive cells in the femur of the broiler (Mroczek-Sosnowska et al., 2017). Copper loaded chitosan nanoparticles also resulted in an increase in weight gain in broilers (Wang et al., 2011). CuNP has also resulted in an increased breast and relative organs weight in broilers (Scott et al., 2018b). We hypothesized that due to their physiochemical properties; copper nanoparticles will be highly absorbed from the small intestine and will improve in body weight gain, thickness of muscle fibers and bone development.

The study was therefore designed to determine the effect of CuNP on growth performance, muscle and bone characteristics and blood biochemical profiles in broiler chicken.

Materials and Methods

The experiment was carried out following the guidelines of Ethical Review Committee, Directorate of Advanced Studies, University of Veterinary and Animal Sciences, Lahore, Pakistan (no. DAS/ 507).

Copper nanoparticles

Copper nanoparticles (25nm size and 99.8% purity) were obtained from skySpring (Nanomaterials Inc., USA). Copper nanoparticles were added in the diet by mixing copper nanoparticles with wheat starch. Nanoparticles and starch were suspended in water and then mixed, dried and mixed with feed.

Experimental design and diets

Three hundred and twenty (n=320) one-day old Hubbard broiler chicken were obtained from a local hatchery. The birds were randomly divided into four groups (n=80) with eight replicates (n= 10) per group. The birds were reared on a rice husk litter with a stocking density of 12 birds/m^2 for all the groups.

A corn-based diet (Table 1) was used during the experiment and was formulated according to the National Research Council recommendations (NRC, 1994) for starter and grower phases. Birds in control group were fed with the basal diet while, birds in other three groups received CuNP mixed in diet at the dose rate of 5mg/kg (A), 10mg/kg (B) and 15mg/kg (C), respectively. Birds were fed *ad libitum* and had free access to the water.

The temperature of the experimental shed was kept at 35 °C with a relative humidity of 65 ± 5%. Temperature was reduced to 3°C every week until it reached 26°C and was maintained until the end of the experiment. Light was supplied for 24 h during the experimental period.

Growth performance

Body weight (BW) and feed conversion ratio (FCR) were recorded on a weekly basis whereas feed intake (FI) was recorded on a daily basis and it was used to calculate feed conversion ratio (FCR).

Blood sampling

At the end of the experiment (35th day), two birds from each replicate were selected and 5 ml blood samples was collected from the right brachial vein with non-heparinized tubes. After centrifugation at 3500 rpm for 15 minutes, serum was separated and was stored at −20°C for analysis. Total protein, cholesterol, glucose, uric acid and alkaline phosphatase were determined using commercially diagnostic kits (Tahir et al., 2019). Concentration of minerals was estimated using atomic absorption spectrophotometer

Table 1. Ingredients and chemical composition of basal diets.

Ingredients (%)	Starter Phase	Grower Phase
Corn	40	57
Rice Broken	15	0
Rice Polish	0	4.5
Wheat Bran	1.2	0
Soybean meal	11	10
Sunflower meal	12	13
Canola meal	9.3	5
Rapeseed meal	5	6
Guar meal	1	0
Molasses	2	1
Di-calcium phosphate	1.76	2
Sodium chloride	0.21	0.41
Sodium bicarbonate	0.03	0.09
Vitamin Mineral Premix	1	1
Vitamin Mineral Premix Composition (per kg)		
Calcium	190 g	190 g
Potassium	80 g	80 g
Sodium	20 g	20 g
Magnesium	8 g	8 g
Zinc	3.5 g	3.5 g
Iron	7.5 g	7.5 g
Manganese	6 g	6 g
Selenium	12 mg	12 mg
Cobalt	40 mg	40 mg
Iodine	35 mg	35 mg
Copper	8.5 g	8.5 g
Vitamin A	200,000 IU	200,000 IU
Vitamin D3	75,000 IU	75,000 IU
Vitamin E	1100 IU	1100 IU
Vitamin K3	30 mg	30 mg
Ascorbic Acid	1400 mg	1400 mg
Thiamine	135 mg	135 mg
Riboflavin	350 mg	350 mg
Niacin	3340 mg	3340 mg
Vitamin B6	350 mg	350 mg
Folic Acid	45 mg	45 mg
Vitamin B12	0.67 mg	0.67 mg
Biotin	13.5 mg	13.5 mg
Chemical composition (% DM)		
Crude protein	19.5	18.5
Crude fat	2.1	2.3
Crude fiber	1.25	1.75
Total Ash	5.7	5.5

(iCE™ 3300, Thermofisher Scientific, USA) as described previously (Yousaf et al., 2009).

pH of muscle

The selected birds were slaughtered, and the right superficial pectoral muscle was used to determine pH. The initial pH (pH_1) was measured by inserting a probe into the muscle using a pH meter (HI 99163). Muscle samples were stored for 24 hours in a refrigerator and final pH (pH_2) was recorded.

Tissue sampling and muscle histomorphometry

For muscle histomorphometry, samples of 2 cm^2 from the left superficial pectoral muscle were collected. Muscle samples were cleaned with saline solution, stored in 10% formalin at room temperature for one week prior to histological processing (Aiyan et al., 2018).Tissues obtained from the left superficial pectoral muscle were processed by paraffin embedding technique. Tissue sections (5µm) were made using a YD-355AT microtome (Jinhua City, Zhejiang Province, China) and stained with Haematoxylin and Eosin (H&E) according to the method described (Bancroft et al., 2018). The diameter of the muscle fiber was determined by the cross section of the H&E stained slides. The muscle fiber diameter was calculated using a software (Progress Cap-ture Pro 2.7.7.; Labomed, USA). Five muscle fibers from three different fascicles were selected and their average was considered as fiber diameter. The cross-sectional area of the muscle fibre was calculated from the diameter of the muscle fiber. Muscle fibers number was counted and muscle fiber number per unit area (muscle fibers/mm^2) was calculated.

Bone characteristics

The left tibia was collected, boiled at 100 °C to remove all muscle matter. Bones were kept at room temperature until dried and were weighed. Length, diaphysis diameter from mid-point was measured using a Vernier caliper. Medullary canal diameter was measured after breaking the bone.

The bone weight/length index, robusticity index and tibiotarsal index were determined using the following formulae, respectively (Kocabağli, 2001) (Equations 1-3).

$$Weight\ /\ length\ index\ =\ weight\ (mg)\ /\ length\ (mm) \tag{1}$$

$$Robusticity\ index\ =\ bone\ length\ (mm)\ /\ cube\ root\ of\ bone\ weight\ (mg) \tag{2}$$

$$Tibiotarsal\ index\ =\ \left(diaphysis\ diameter\ -\ medullary\ canal\ diameter\right)\ /\ diaphysis\ diameter\ \times\ 100 \tag{3}$$

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Statistical Packages for Social Sciences software (SPSS Inc. Version 20, Chicago IL, USA). The mean difference was calculated using Duncan's multiple range tests and results were considered significant at P≤0.05. Orthogonal polynomial contrasts were used to examine linear and quadratic effects of different inclusion levels CuNP.

Results

Growth performance

The average body weight (BW), total feed intake (TFI), feed conversion ratio (FCR) and average daily weight gain (ADWG) is shown in Table 2. The results show that adding CuNP to the broiler diets linearly improved BW, ADWG and TFI in broilers ($P \leq 0.05$). The highest BW, ADWG and highest TFI were recorded with CuNP at 15mg/kg followed by B, A and control group respectively. Inclusion of CuNP in broiler diets linearly decreased FCR ($P \leq 0.05$). Highest FCR (1.74 ±0.02) was observed in control group followed by A, B and C groups respectively. CuNP supplementation also showed a quadratic increase on BW, TFI and ADWG.

Blood analysis

Results from the serum analysis of broiler is shown in Table 3. The level of total pro-tein and globulin in blood increased linearly ($P \leq 0.05$) by CuNP inclusion in diets. The concentration of glucose and uric acid decreased linearly ($P \leq 0.05$) with CuNP supple-mentation in diets. Copper nanoparticles supplementation has no linear or quadratic effect on albumin, cholesterol and ALP levels in the blood ($P>0.05$).

Tibia bone characteristics

The effects of different levels of CuNP on tibia bone are summarized in Table 4. Weight, length, diaphysis diameter, medullary canal diameter, weight/length index and tibiotarsal index linearly increased ($P \leq 0.05$) with CuNP supplementation. However, there was no linear effect of CuNP on robusticity index of tibia bone ($P>0.05$). Copper nanoparticles supplementation also showed a quadratic increase in bone weight, DD, MCD and W/L index ($P \leq 0.05$).

Muscle characteristics

The effects of CuNP on selected characteristics of pectoral muscles are summarized in Table 5. A linear increase ($P \leq 0.05$) in muscle pH was observed during both determinations in the birds. The muscle fiber diameter, muscle fascicle diameter, cross sectional area of the muscle and fascicle increased linearly ($P \leq 0.05$) with CuNP supplementation in diet. Copper nanoparticles inclusion in broiler diets resulted in reduction of muscle fiber numbers ($P \leq 0.05$).

Mineral concentration in blood

The concentration of different minerals in blood is summarized in Table 6. A significant linear increase ($P \leq 0.05$) in copper, calcium and phosphorus was observed in all the treated groups as compared to the control group. Fe, Ca and P also showed a quadratic increase ($P \leq 0.05$) with CuNP supplementation in diet.

Discussion

The results of the present study indicated that the final body weight and FCR were improved using of the copper nanoparticles. An improved growth performance

Table 2. Effect of dietary supplementation of copper nanoparticles (CuNP) on performance parameters of 35 days old broilers.

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
BW (g)	1568±15^{d}	1637±12^{c}	1645±12^{b}	1760±19^{a}	<0.001	<0.001	0.020
TFI (g)	2741±5^{d}	2735±6^{c}	2756±5^{b}	2775±5^{a}	<0.001	<0.001	< 0.001
FCR	1.74±0.02^{a}	1.67±0.01^{b}	1.67±0.02^{b}	1.57±0.06^{c}	<0.001	<0.001	0.126
ADWG (g)	44.8±0.4^{c}	46.7±0.3^{b}	47.0±0.6^{b}	47.0±0.6^{b}	<0.001	<0.001	0.020

"a-d" means within a row with different superscripts differ significantly ($P \leq 0.05$). Control, Basal diet. A, basal diet + 5 mg/kg CuNP. B, basal diet + 10 mg/kg CuNP. C, basal diet + 15 mg/kg CuNP. BW, Final body weight. TFI, total feed intake (g/bird/). ADWG, average daily gain (g/bird/ day). FCR, feed conversion ratio.

Table 3. Effects of copper nanoparticles (CuNP) supplementation on serum biochemical indices in broilers.

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
Total Proteins (g/dl)	3.3±0.5^{b}	3.1±0. 6^{b}	4.1±0.3^{a}	4.0±0.5^{a}	0.002	0.017	0.097
Albumin (g/dl)	1.4±0.4^{a}	0.9±0.2^{b}	1.2±0.3^{ab}	1.0±0.2^{b}	0.032	0.113	0.331
Globulin (g/dl)	1.8±0.1^{b}	2.2±0.6^{b}	2.8±0.5^{a}	2.9±0.3^{a}	<0.001	<0.001	0.436
Cholesterol(mg/dl)	123.±34^{a}	118±12^{a}	88±3^{b}	65±3^{c}	<0.001	0.071	0.152
Glucose (mg/dl)	304±5^{a}	276±8^{b}	254±3^{c}	226±12^{d}	<0.001	<0.001	<0.001
Uric Acid (mg/dl)	5.4±0.6^{a}	5.4±0.2^{a}	4.6±1.5^{b}	3.2±1.3^{c}	<0.001	0.003	0.147
ALP (IU/L)	3007±746^{a}	2867±620^{a}	2246±184^{b}	2097±395^{b}	0.004	0.006	0.988

"a-c" means within a row with different superscripts differ significantly ($P \leq 0.05$). ALP, Alkaline Phosphatase. Control, Basal diet. A, basal diet + 5 mg/kg CuNP. B, basal diet + 10 mg/kg CuNP. C, basal diet + 15 mg/kg CuNP.

Table 4. Effects of copper nanoparticles (CuNP) supplementation on tibia bone characteristics in broilers.

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
Bone Weight (mg)	3970 ±65^{c}	4280±80^{b}	4355±90^{b}	5366±144^{a}	<0.001	<0.001	< 0.001
Bone Length(mm)	87.1±3.5^{b}	88.9±1.6^{b}	88.6±2.4^{b}	92.8±3.0^{a}	<0.001	0.02	0.16
DD(mm)	7.1±0.1^{c}	7.3±0.1^{b}	7.2±0.2^{bc}	8.7±0.3^{a}	<0.001	<0.001	<0.001
MCD(mm)	3.7±0.8^{c}	3.9±0.7^{b}	3.8±0.8^{bc}	4.5±0.1^{a}	<0.001	<0.001	<0.001
W/L index	45.6±2.4^{c}	48.1±2.7^{b}	49.1±2.2^{b}	58.2±2.8^{a}	<0.001	<0.001	<0.001
R. Index	5.5±10.2^{a}	5.4±0.8^{a}	5.4±0.1^{ab}	5.2±0.1^{b}	0.04	0.062	0.13
TT index	45.8±1.3^{b}	46.0±1.6^{b}	46.8±1.2^{ab}	48.1±2.1^{a}	0.03	0.036	0.45

"a-c" means within a row with different superscripts differ significantly ($P \leq 0.05$). Control, Basal diet. A, basal diet + 5 mg/kg CuNP. B, basal diet + 10 mg/kg CuNP. C, basal diet + 15 mg/kg CuNP. DD; Diaphysis diameter, MCD; Medullary canal diameter, W/L; Weight/ Length index, R. Index; Robusticity index, TT index; Tibiotarsal index.

Table 5. Effects of copper nanoparticles (CuNP) supplementation on pectoral muscle characteristics in broilers.

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
Fibre diameter(µm)	44.4 ±2.2^{d}	47.1±1.0^{c}	53.8±1.5^{b}	55.6±1.6^{a}	<0.001	<0.001	0.35
FCSA (µm)^{2}	1987±143^{d}	2284±165^{c}	2666±280^{b}	2726±268^{a}	<0.001	<0.001	0.002
Fascicle diameter(mm)	0.82±0.03^{d}	0.94±0.08^{c}	1.03±0.05^{b}	1.05±0.06^{a}	<0.001	<0.001	0.025
FaCSA(mm)^{2}	0.54±0.02^{d}	0.69±0.03^{c}	0.93±0.04^{b}	0.95±0.05^{a}	<0.001	<0.001	<0.001
Fibre No/mm^{2}	528±22^{a}	475±25^{bc}	491±24^{b}	463±19^{c}	<0.001	0.002	0.053
pH_1	6.21±0.02^{c}	6.21±0.03^{c}	6.42±0.06^{b}	6.49±0.05^{a}	<0.001	<0.001	0.24
pH_2	5.91±0.08^{c}	6.06±0.05^{c}	6.05±0.02^{bc}	6.13±0.05^{a}	<0.001	<0.001	0.01

"a-c" means within a row with different superscripts differ significantly ($P \leq 0.05$). pH1, initial pH; pH2, final pH; FCSA, Fibre cross section area; FaSCA, Fascicle cross section area. Control, Basal diet. A, basal diet + 5 mg/kg CuNP. B, basal diet + 10 mg/kg CuNP. C, basal diet + 15 mg/kg CuNP.

Table 6. Effects of copper nanoparticles (CuNP) supplementation on blood minerals concentration.

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
Fe (µg/ml)	2.96±0.05^{b}	2.94±0.03^{b}	2.97±0.001^{b}	3.5±0.001^{a}	<0.001	<0.001	<0.001
Cu (µg/ml)	0.28±0.03^{c}	0.32±0.02^{b}	0.33±0.04^{b}	0.37±0.03^{a}	<0.001	<0.001	0.61
Ca (mg/dl)	5.6±0.04^{d}	6.3±0.09^{c}	6.5±0.07^{b}	6.9±0.12^{a}	<0.001	<0.001	0.010
P (mg/dl)	9.6±0.1^{c}	10.6±0.2^{b}	10.8±0.2^{b}	11.0±0.8^{a}	<0.001	<0.001	<0.001

"a-d" means within a row with different superscripts differ significantly ($P \leq 0.05$). Fe, Iron; Cu, Copper; Ca, Calcium; P, Phosphorus. Control, Basal diet. A, basal diet + 5 mg/kg CuNP. B, basal diet + 10 mg/kg CuNP. C, basal diet + 15 mg/kg CuNP.

was also observed in broilers by Wang et al. (2011) using copper loaded chitosan nanoparticles at 50 and 100mg/kg. Final body weight of the broiler also increased using copper nanoparticles (Sawosz et al., 2018). El-Kazaz and Hafez (2019) also reported an increase in the body weight of broilers using copper nanoparticles in drinking water. An improvement in broiler performance was also observed as a result of intramuscular injections of copper nanoparticles (Miroshnikov et al., 2015). In ovo treatment of copper nanoparticles also resulted in an improved body weight gain and FCR in broilers (Scott et al., 2018b). Positive results were also observed on the growth performance of pigs (Wang et al., 2012), rabbits (Refaie et al., 2015) fish (El-Basuini et al., 2016) and with inclusion of copper nanoparticles in diet.

An increase in broiler growth performance might be due to the higher bioavailability of the copper nanoparticles due to their smaller size and ability to diffuse from gastrointestinal tract and blood absorption (Singh, 2016). Copper nanoparticles inhibit growth of several bacteria like *S. aureus, B. subtilis* and *E. coli* bacteria (Shobha et al., 2014), *Micrococcus luteus, Klebsiellapneumoniae and*

Pseudomonas aeruginosa (Ramyadevi et al., 2012) and this might have helped in minimizing the bacterial burden in broilers which helped to utilize the diet properly and resulted in an increased growth performance of broiler. Copper nanoparticles also reduce the fermentation loss of nutrients by altering gut microbiota (Højberg et al., 2005). In addition to this, antioxidant properties of copper might have helped in improving performance of broiler as copper acts as an antioxidant agent in the diet (Pineda et al., 2013).

Blood biochemical indices maintain several homeostatic events in the body and are an important indicators of broilers health status. In our study, supplementation of copper nanoparticles at 15 mg/kg decreased concentrations of glucose, uric acid, and alkaline phosphatase. These results agree with Kumar et al. (2013), who reported a decrease in the concentration of cholesterol, glucose and alkaline phosphatases but observed an increase in uric acid by the use of copper in broiler diets. A decrease in the concentration of glucose and cholesterol was also observed by Mroczek-Sosnowska et al. (2015) as a result of in ovo injections of copper nanoparticles. Scott et al. (2018b) observed a decreased concentration glucose and using copper nanoparticles in broiler chicken. Supplementation of copper nanoparticles also decreased the concentration of glucose in fish (Basuini et al., 2016).

Several other studies (Kumar et al., 2013; Payvastegan et al., 2013; Zahedi et al., 2013) have also reported a decrease in the concentration of cholesterol using copper in broilers diets. The decrease in the cholesterol levels might be due to the indirect role of copper in cholesterol biosynthesis by increasing the oxidized form of glutathione (Kim et al., 1992). Low levels of alkaline phosphatase is an indicator of healthy bones as higher alkaline phosphatase level determines bone weakness (Kocabağli, 2001).

A decreased concentration of uric acid and a higher concentration of total protein was observed in copper nanoparticles treated groups as compared to the control group respectively, which can be attributed by the fact that amino acids were, used efficiently for protein synthesis (Yang et al., 2009). An increased blood protein concentration was also observed by using copper loaded chitosan nanoparticles in feed (Wang et al., 2011). This might be an indicator that protein synthesis is enhanced using copper nanoparticles (Scott et al., 2018b).

The tibia is a long bone which helps in supporting the weight of the birds and provides strength to the musculoskeletal system. Bone length, weight and breaking strength are an important indicator of bone strength (Sahraei et al., 2012). In current study, weight, length, diameter, weight/length index and tibiotarsal index increased using copper nanoparticles as compared to the control group.

The bones will be denser when robusticity index is low and tibiotarsal index and weight/ length index is high (Kocabağli, 2001). The results of the current study are similar to the findings of Kwiecień et al. (2015) who observed an increase in weight, length and circumference of femur bones in copper fed birds. Banks et al. (2004) also observed an increase in weight of the tibia bone when using copper lysine in the diet. Mroczek-Sosnowska et al. (2017) found out that in ovo copper nanoparticles resulted in higher weight and volume of the femoral bone in broiler chicken and such bones were more resistant to fractures. Copper nanoparticles also improved the bone density in rats as compared to copper carbonate (Tomaszewska et al., 2017). Additionally, lower level of serum alkaline phosphatase level in copper treated groups indicate that the bones are stronger as higher serum alkaline phosphatase level is associated with lower bone density (Kang et al., 2015).

These results can be attributed to the fact that dietary CuNP influenced the synthesis of collagen fibers, which are essential for bone strength and density (McNerny et al., 2015). In addition to this, proper calcium and phosphorus might have been deposited in the bone by the action of action of lysyl oxidase (a copper dependent enzyme) in copper supplemented groups (Nguyen et al., 2022).

In current study, the characteristics of the pectoralis muscle fiber were improved significantly in copper supplemented groups. Similarly, an improvement in the skeletal muscles growth was observed as a result of copper supplementation in rabbits (Lei et al., 2017). In ovo supplementation of copper nanoparticles resulted in a higher percentage breast and leg muscles (Mroczek-Sosnowska et al., 2016).

A higher pH2 was recorded in muscles of copper supplemented groups as compared to the control group. A higher muscle pH is an indicator of meat tenderness (Li et al., 2014). This may be due to the antioxidant effect of the copper (Pineda et al., 2013). Literature on the effects of copper nanoparticles on the muscles characteristics is rare. An increase in fascicle diameter and fascicle cross-sectional area may be due to the higher relevant muscle fiber diameter and cross sectional area. This may be attributed to the role of copper in muscle development in broiler chickens (Mroczek-Sosnowska et al., 2015) and due to the involvement of copper in collagen formation (McNerny et al., 2015).

In current study, an increase in blood concentration of iron, copper, calcium, and phosphorus was observed in copper supplemented groups. These results agree with the findings of (Mroczek-Sosnowska et al., 2013) who reported an increase level of calcium, phosphorus and iron in blood of broilers as a result of in ovo supplementation of copper nanoparticles. An increased level of copper and iron was also observed by the use of copper in different concentration in broilers (Samanta et al., 2011). However, Scott et al. (2018b) and Wang et al. (2011) did not observe a significant difference in the concentration of calcium and phosphorus in blood when they used copper nanoparticles and chitosan loaded copper nanoparticles in broilers. An in-crease in concentration of serum copper was observed using organic copper in pigs (Close, 1998). A higher concentration of copper in blood, heart, liver, lung and bone was also observed by the use of organic copper in broilers as compared to the inorganic form of copper (Jegede et al., 2011).

The increase in the concentration of blood iron and copper in supplemented groups may be due to the role of copper and iron in synthesis of hemoglobin (Samanta et al., 2011). The increased level of calcium and phosphorus in treated groups might be due to the action of the copper nanoparticles on lysyl oxidase enzymes in nanoparticles supplemented groups (McNerny et al., 2015).

The results of the current study suggest that dietary copper nanoparticles at 15mg/kg resulted in higher weight gain and improved FCR in broilers. In addition to this, it has also improved muscle and bone characteristics in broilers.

References

AIYAN, A.A., RICHARDSON, K., SHAWAF, T., ABDULLAH, S. and BARIGYE, R., 2018. A histologic and histomorphometric study of the first compartment of stomach in the dromedary (*Camelus dromedarius*). *Journal of Camel Practice and Research*, vol. 25, no. 2, pp. 221-230. http://dx.doi.org/10.5958/2277-8934.2018.00029.2.

BANCROFT, J.D., FLOYD, A.D. and SUVARNA, S.K., 2018. *Bancroft's theory and practice of histological techniques*. 8th ed. New York: Elsevier.

BANKS, K.M., THOMPSON, K.L., RUSH, J.K. and APPLEGATE, T.J., 2004. Effects of copper source on phosphorus retention in broiler chicks and laying hens. *Poultry Science*, vol. 83, no. 6, pp. 990-996. http://dx.doi.org/10.1093/ps/83.6.990. PMid:15206627.

BASUINI, M.F., EL-HAIS, A.M., DAWOOD, M.A.O., ABOU-ZEID, A.E.-S., EL-DAMRAWY, S.Z., KHALAFALLA, M.M.E.-S., KOSHIO, S., ISHIKAWA, M. and DOSSOU, S., 2016. Effect of different levels of dietary copper nanoparticles and copper sulfate on growth performance, blood biochemical profiles, antioxidant status and immune response of red sea bream Pagrus major. *Aquaculture*, vol. 455, pp. 32-40. http://dx.doi.org/10.1016/j.aquaculture.2016.01.007.

BLAKE, G.M. and FOGELMAN, I., 2002. Methods and clinical issues in bone densitometry and quantitative ultrasonometry. In: J.P. BILEZIKIAN, L.G. RAISZ and G.A. RODAN, eds. *Principles of bone biology*. San Diego, Academic Press, vol. 2, pp. 1573-1585. http://dx.doi.org/10.1016/B978-012098652-1/50193-1.

BOGUCKA, J., RIBEIRO, D.M., COSTA, R.P.R. and BEDNARCZYK, M., 2018. Effect of synbiotic dietary supplementation on histological and histopathological parameters of Pectoralis major muscle of broiler chickens. *Czech Journal of Animal Science*, vol. 63, no. 7, pp. 263-271. http://dx.doi.org/10.17221/103/2017-CJAS.

CLOSE, W.H., 1998. The role of trace mineral proteinates in pig nutrition. *Biotechnology in the Feed Industry*, vol. 14, pp. 469-474.

EL-KAZAZ, S.E. and HAFEZ, M.H., 2019. Evaluation of copper nanoparticles and copper sulfate effect on immune status, behavior, and productive performance of broilers. *Journal of Advanced Veterinary and Animal Research*, vol. 7, no. 1, pp. 16-25. PMid:32219105.

GAU, J.-T., CHAVAN, B., LI, Y., CLARK, B.C. and HAILE, Z.T., 2021. Association between serum zinc levels and basic physical functioning: secondary data analysis of NHANES 2011-14. *BMC Nutrition*, vol. 7, no. 1, p. 57. http://dx.doi.org/10.1186/s40795-021-00461-z. PMid:34629117.

GENCHEV, A., MIHAYLOVA, G., RIBARSKI, S., PAVLOV, S. and KABAKCHIEV, S., 2008. Meat quality and composition in Japanese quails. *Trakia Journal of Science*, vol. 64, pp. 72-82.

HØJBERG, O., CANIBE, N., POULSEN, H.D., HEDEMANN, M.S. and JENSEN, B.B., 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Applied and Environmental Microbiology*, vol. 71, no. 5, pp. 2267-2277. http://dx.doi.org/10.1128/AEM.71.5.2267-2277.2005. PMid:15870311.

JEGEDE, A.V., ODUGUWA, O.O., BAMGBOSE, A.M., FANIMO, A.O. and NOLLET, L., 2011. Growth response, blood characteristics and copper accumulation in organs of broilers fed on diets supplemented with organic and inorganic dietary copper sources. *British Poultry Science*, vol. 52, no. 1, pp. 133-139. http://dx.doi.org/10.1080/00071668.2010.544714. PMid:21337208.

KANG, K.Y., HONG, Y.S., PARK, S.-H. and JU, J.H., 2015. Increased serum alkaline phosphatase levels correlate with high disease activity and low bone mineral density in patients with axial spondyloarthritis. *Seminars in Arthritis and Rheumatism*, vol. 45, no. 2, pp. 202-207. http://dx.doi.org/10.1016/j.semarthrit.2015.03.002. PMid:25895696.

KIM, S., CHAO, P.Y. and ALLEN, G.D., 1992. Inhibition of elevated hepatic glutathione abolishes copper deficiency cholesterolemia. *The FASEB Journal*, vol. 6, no. 7, pp. 2467-2471. http://dx.doi.org/10.1096/fasebj.6.7.1563598. PMid:1563598.

KOCABAĞLI, N., 2001. The effect of dietary phytase supplementation at different levels on tibial bone characteristics and strength in broilers. *Turkish Journal of Veterinary and Animal Sciences*, vol. 25, pp. 797-802.

KUMAR, P., BISWAS, A., BHARTI, V.K. and SRIVASTAVA, R.B., 2013. Effects of dietary copper supplementation on performance and blood biochemical parameters in broiler chickens at cold desert region in India. *The Journal of Veterinary Science Photon*, vol. 114, pp. 166-172.

KWIECIEŃ, M., WINIARSKA-MIECZAN, A., ZAWIŚLAK, K. and SROKA, S., 2015. Effect of copper glycinate chelate on biomechanical, morphometric and chemical properties of chicken femur. *Annals of Animal Science*, vol. 14, no. 1, pp. 127-139. http://dx.doi.org/10.2478/aoas-2013-0085.

LEI, L., XIAOYI, S. and FUCHANG, L., 2017. Effect of dietary copper addition on lipid metabolism in rabbits. *Food & Nutrition Research*, vol. 61, no. 1, p. 1348866. http://dx.doi.org/10.1080/16546628.2017.1348866. PMid:28747869.

LI, P., WANG, T., MAO, Y., ZHANG, Y., NIU, L., LIANG, R., ZHU, L. and LUO, X., 2014. Effect of ultimate pH on postmortem myofibrillar protein degradation and meat quality characteristics of Chinese yellow crossbreed cattle. *The Scientific World Journal*, vol. 2014, p. 174253. PMid:25197695.

MCNERNY, E.M., GONG, B., MORRIS, M.D. and KOHN, D.H., 2015. Bone fracture toughness and strength correlate with collagen cross-link maturity in a dose-controlled lathyrism mouse model. *Journal of Bone and Mineral Research*, vol. 30, no. 3, pp. 455-464. http://dx.doi.org/10.1002/jbmr.2356. PMid:25213475.

MIGGIANO, G.A.D. and GAGLIARDI, L., 2005. Diet, nutrition and bone health. *La Clinica Terapeutica*, vol. 156, no. 1-2, pp. 47-56. PMid:16080661.

MIROSHNIKOV, S.A., YAUSHEVA, E.V., SIZOVA, E.A. and MIROSHNIKOVA, E.P., 2015. Comparative assessment of effect of copper nano-and microparticles in chicken. *Oriental Journal of Chemistry*, vol. 31, pp. 2327-2336. http://dx.doi.org/10.13005/ojc/310461.

MROCZEK-SOSNOWSKA, N., BATORSKA, M., LUKASIEWICZ, M., WNUK, A., SAWOSZ, E., JAWORSKI, S. and NIEMIEC, J., 2013. Effect of nanoparticles of cop-per and copper sulfate administered in ovo on hematological and biochemical blood markers of broiler chickens. *Annals of Warsaw University of Life Sciences*, vol. 52, pp. 141-149.

MROCZEK-SOSNOWSKA, N., ŁUKASIEWICZ, M., ADAMEK, D., KAMASZEWSKI, M., NIEMIEC, J., WNUK-GNICH, A., SCOTT, A., CHWALIBOG, A. and SAWOSZ, E., 2017. Effect of copper nanoparticles administered *in ovo* on the activity of proliferating cells and on the resistance of femoral bones in broiler chickens.

Archives of Animal Nutrition, vol. 71, no. 4, pp. 327-332. http://dx.doi.org/10.1080/1745039X.2017.1331619. PMid:28581358.

MROCZEK-SOSNOWSKA, N., ŁUKASIEWICZ, M., WNUK, A., SAWOSZ, E., NIEMIEC, J., SKOT, A., JAWORSKI, S. and CHWALIBOG, A., 2016. *In ovo* administration of copper nanoparticles and copper sulfate positively influences chicken performance. *Journal of the Science of Food and Agriculture*, vol. 96, no. 9, pp. 3058-3062. http://dx.doi.org/10.1002/jsfa.7477. PMid:26417698.

MROCZEK-SOSNOWSKA, N., SAWOSZ, E., VADALASETTY, K.P., LUKASIEWICZ, M., NIEMIEC, J., WIERZBICKI, M., KUTWIN, M., JAWORSKI, S. and CHWALIBOG, A., 2015. Nanoparticles of copper stimulate angiogenesis at systemic and molecular level. *International Journal of Molecular Sciences*, vol. 16, no. 3, pp. 4838-4849. http://dx.doi.org/10.3390/ijms16034838. PMid:25741768.

NATIONAL RESEARCH COUNCIL - NRC, 1994. Nutrient requirements of poultry 9th rev. ed. Washington, DC: National Academy Press.

NGUYEN, H.T.T., KHERAVII, S.K., WU, S.B., ROBERTS, J.R., SWICK, R.A. and TOGHYANI, M., 2022. Sources and levels of copper affect liver copper profile, intestinal morphology and cecalmicrobiota population of broiler chickens fed wheat-soybean meal diets. *Scientific Reports*, vol. 12, no. 1, p. 2249. http://dx.doi.org/10.1038/s41598-022-06204-9. PMid:35145167.

ORBAN, J.I., ADEOLA, O. and STROSHINE, R., 1999. Microbial phytase in finisher diets of white Pekin ducks: effects on growth performance, plasma phosphorus concentration, and leg bone characteristics. *Poultry Science*, vol. 78, no. 3, pp. 366-377. http://dx.doi.org/10.1093/ps/78.3.366. PMid:10090263.

PALACIOS, C., 2006. The role of nutrients in bone health, from A to Z. *Critical Reviews in Food Science and Nutrition*, vol. 46, no. 8, pp. 621-628. http://dx.doi.org/10.1080/10408390500466174. PMid:17092827.

PANG, Y., PATTERSON, J. and APPLEGATE, T., 2009. The influence of copper concentration and source on ilealmicrobiota. *Poultry Science*, vol. 88, no. 3, pp. 586-592. http://dx.doi.org/10.3382/ps.2008-00243. PMid:19211529.

PAYVASTEGAN, S., FARHOOMAND, P. and DELFANI, N., 2013. Growth performance, organ weights and blood parameters of broilers fed diets containing graded levels of dietary canola meal and supplemental copper. *Poultry Science Journal*, vol. 50, no. 4, pp. 354-363. http://dx.doi.org/10.2141/jpsa.0130006.

PINEDA, L., SAWOSZ, E., VADALASETTY, K.P. and CHWALIBOG, A., 2013. Effect of copper nanoparticles on metabolic rate and development of chicken embryos. *Animal Feed Science and Technology*, vol. 186, no. 1-2, pp. 125-129. http://dx.doi.org/10.1016/j.anifeedsci.2013.08.012.

RATH, N.C., HUFF, G.R., HUFF, W.E. and BALOG, J.M., 2000. Factors regulating bone maturity and strength in poultry. *Poultry Science*, vol. 79, no. 7, pp. 1024-1032. http://dx.doi.org/10.1093/ps/79.7.1024. PMid:10901206.

REFAIE, A., GHAZAL, M., BARAKAT, S., MORSY, W., MESHREKY, S., YOUNAN, G. and EISA, W., 2015. Nano-copper as a new growth promoter in the diet of growing New Zealand white rabbits. *Egyptian Journal of Rabbit Science*, vol. 25, no. 1, pp. 39-57. http://dx.doi.org/10.21608/ejrs.2015.46697.

REHFELDT, C. and KUHN, G., 2006. Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. *Journal of Animal Science*, vol. 84, suppl. 13, pp. E113-E123. http://dx.doi.org/10.2527/2006.8413_supplE113x. PMid:16582082.

SAHRAEI, M., JANMMOHAMDI, H., TAGHIZADEH, A. and CHERAGHI, S., 2012. Effect of different zinc sources on tibia bone morphology and ash content of broiler chickens. *Advance Biological Research*, vol. 63, pp. 128-132.

SAMANTA, B., GHOSH, P.R., BISWAS, A. and DAS, S.K., 2011. The effects of copper supplementation on the performance and hematological parameters of broiler chickens. *Asian-Australasian Journal of Animal Sciences*, vol. 24, no. 7, pp. 1001-1006. http://dx.doi.org/10.5713/ajas.2011.10394.

SAWOSZ, E., ŁUKASIEWICZ, M., ŁOZICKI, A., SOSNOWSKA, M., JAWORSKI, S., NIEMIEC, J., SCOTT, A., JANKOWSKI, J., JÓZEFIAK, D. and CHWALIBOG, A., 2018. Effect of copper nanoparticles on the mineral content of tissues and droppings, and growth of chickens. *Archives of Animal Nutrition*, vol. 72, no. 5, pp. 396-406. http://dx.doi.org/10.1080/1745039X.2018.1505146. PMid:30183391.

SCOTT, A., VADALASETTY, K.P., CHWALIBOG, A. and SAWOSZ, E., 2018a. Copper nanoparticles as an alternative feed additive in poultry diet, a review. *Nanotechnology Reviews*, vol. 7, no. 1, pp. 69-93. http://dx.doi.org/10.1515/ntrev-2017-0159.

SCOTT, A., VADALASETTY, K.P., LUKASIEWICZ, M., JAWORSKI, S., WIERZBICKI, M., CHWALIBOG, A. and SAWOSZ, E., 2018b. Effect of different levels of copper nanoparticles and copper sulphate on performance, metabolism and blood biochemical profiles in broiler chicken. *Journal of Animal Physiology and Animal Nutrition*, vol. 102, no. 1, pp. e364-e373. http://dx.doi.org/10.1111/jpn.12754. PMid:28608638.

SHEN, Q., QI, Y., KONG, Y., BAO, H., WANG, Y., DONG, A., WU, H. and XU, Y., 2022. Advances in copper-based biomaterials with antibacterial and Os-teogenic properties for bone tissue engineering. *Frontiers in Bioengineering and Biotechnology*, vol. 9, p. 795425. http://dx.doi.org/10.3389/fbioe.2021.795425. PMid:35127670.

SHOBHA, G., MOSES, V. and ANANDA, S., 2014. Biological synthesis of copper nanoparticles and its impact: a review. *International Journal of Pharmaceutical Science Invention*, vol. 3, no. 8, pp. 28-38.

SINGH, P., 2016. Use of nano feed additives in livestock feeding. *International Journal of Livestock Research*, vol. 6, no. 1, pp. 1-14. http://dx.doi.org/10.5455/ijlr.20150816121040.

SOETAN, K.O., OLAIYA, C.O. and OYEWOLE, O.E., 2010. The importance of mineral elements for humans, domestic animals, and plants, a review. *African Journal of Food Science*, vol. 4, pp. 200-222.

TAHIR, S.K., YOUSAF, M.S., RASHID, M.A., KHAN, A.F., AHMAD, S., ZANEB, H., KHAN, I. and REHMAN, H., 2019. Supplemental chromium-loaded chitosan nanoparticles affect growth, serum metabolites and intestinal histology in broilers. *South African Journal of Animal Science*, vol. 49, no. 6, pp. 1072-1082. http://dx.doi.org/10.4314/sajas.v49i6.11.

TOBA, Y., KAJITA, Y., MASUYAMA, R., TAKADA, Y., SUZUKI, K. and AOE, S., 2000. Dietary magnesium supplementation affects bone metabolism and dynamic strength of bone in ovariectomized rats. *The Journal of Nutrition*, vol. 130, no. 2, pp. 216-220. http://dx.doi.org/10.1093/jn/130.2.216. PMid:10720172.

TOMASZEWSKA, E., MUSZYŃSKI, S., OGNIK, K., DOBROWOLSKI, P., KWIECIEŃ, M., JUŚKIEWICZ, J., CHOCYK, D., ŚWIETLICKI, M., BLICHARSKI, T. and GŁADYSZEWSKA, B., 2017. Comparison of the effect of dietary copper nanoparticles with copper II salt on bone geometric and structural parameters as well as material characteristics in a rat model. *Journal of Trace Elements in Medicine and Biology*, vol. 42, pp. 103-110. http://dx.doi.org/10.1016/j.jtemb.2017.05.002. PMid:28595781.

WANG, C., WANG, M., YE, S., TAO, W. and DU, Y., 2011. Effects of copper loaded chitosan nanoparticles on growth and immunity in broilers. *Poultry Science*, vol. 90, no. 10, pp. 2223-2228. http://dx.doi.org/10.3382/ps.2011-01511. PMid:21934004.

WANG, M.Q., DU, Y.J., WANG, C., TAO, W.J., HE, Y.D. and LI, H., 2012. Effects of copper-loaded chitosan nanoparticles on intestinal microflora and morphology in weaned piglets. *Biological Trace Element Research*, vol. 149, no. 2, pp. 184-189. http://dx.doi.org/10.1007/s12011-012-9410-0. PMid:22544767.

YANG, Y.X., GUO, J., YOON, S.Y., JIN, Z., CHOI, J.Y., PIAO, X.S., KIM, B.W., OHH, S.J., WANG, M.H. and CHAE, B.J., 2009. Early energy and protein reduction: effects on growth, blood profiles and expression of genes related to protein and fat metabolism in broilers. *British Poultry Science*, vol. 50, no. 2, pp. 218-227. http://dx.doi.org/10.1080/00071660902736706. PMid:19373723.

YOUSAF, M.S., RAHMAN, Z.U., SANDHU, M.A., BUKHARI, S.A. and YOUSAF, A., 2009. Comparison of the fast-induced and high dietary zinc-induced molting, trace elements dynamic in serum and eggs at different production stages in hens *Gallus domesticus. Journal of Animal Physiology and Animal Nutrition*, vol. 93, no. 1, pp. 35-43. http://dx.doi.org/10.1111/j.1439-0396.2007.00775.x. PMid:19386006.

ZAHEDI, M., GHALEHKANDI, J.G., EBRAHIMNEZHAD, Y. and EMAMI, F., 2013. Effects of different levels of copper sulfate on blood biochemical traits in japanese quail *Coturnixcoturnix japonica. International Journal of Biosciences*, vol. 3, no. 12, pp. 221-226. http://dx.doi.org/10.12692/ijb/3.12.221-226.