Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Tetramethylpyrazine Mitigates Toxicity and Liver Oxidative Stress in Tibial Dyschondroplasia Chickens

Khalid Mehmood[1,2§], Hui Zhang[1§], Muhammad Kashif Iqbal[1], Mujeeb Ur Rehman[1], Kun Li[1], Shucheng Huang[1], Muhammad Shahzad[1], Fazul Nabi[1], Mujahid Iqbal[1] and Jiakui Li[1,3,*]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, PR China; [2]College of Veterinary and Animal Sciences, Islamia University of Bahawalpur, Pakistan; [3]College of Animals Husbandry and Veterinary Medicine, Tibet Agricultural and Animal Husbandry University, Linzhi, Tibet 860000, PR China
*Corresponding author: lijk210@sina.com

ARTICLE HISTORY (17-239)

Received: July 10, 2017
Revised: August 23, 2017
Accepted: August 30, 2017
Published online: November 21, 2017

Key words:
Chickens
Liver
Tetramethylpyrazine
Thiram
Tibial dyschondroplasia

ABSTRACT

Tibial dyschondroplasia (TD) is a skeletal abnormality in fast growing bird, which causes down grading and serious poultry welfare issues in meat industry. TD is described as a non-viable and un-mineralized cartilage in the tibial growth plate of chickens which fails to become bone. The present study was designated to check the effect of Tetramethylpyrazine (TMP) on liver oxidative stress and toxicity in thiram induced tibial dyschondroplasia chickens. One hundred and eighty (1-day old) broiler chickens were equally divided into three groups including Control, Thiram induced (50mg/kg/d) and TMP treatment (30 mg/kg/d). All groups were raised under standard hygienic conditions and chickens were slaughtered to collect blood, liver and tibial bone samples on day 7, 10, 14 and 18 from each group. Thiram caused lameness, and significantly decreased ALP activity and increased ALT and AST contents in the serum of thiram group as compared to control group. The level of GSH-Px, T-AOC and SOD was significantly decreased ($P<0.05$), while the MDA value and size of growth plate (GP) was increased in TD afflicted chickens as compared to control group. However, the TMP administration increased the ALP contents, and decreased the AST and ALT contents significantly ($P<0.05$). Furthermore, it increased SOD, T-AOC and GSH-Px and decreased MDA contents significantly ($P<0.05$). TMP treatment to TD afflicted birds prevented lameness, while reinstated the size of growth plate, angiogenesis, and antioxidant imbalance. TMP may be effective to treat and control of TD along with minimizing the liver damages in TD chickens.

To Cite This Article: Mehmood K, Zhang H, Iqbal MK, Rehman MU, Li K, Huang S, Shahzad M, Nabi F, Mujahid I and Li J, xxxx. Tetramethylpyrazine mitigates toxicity and liver oxidative stress in tibial dyschondroplasia chickens. Pak Vet J, xx(x): xxx.

INTRODUCTION

Avian tibial dyschondroplasia disease is common in fast growing chickens round the world in which growth plate/cartilage accumulates in metaphyseal region of tibial bone, which is similar to mammalian osteochondrosis (Orth and Cook, 1994). TD is characterized by avascular and non-mineralize cartilage in the tibial growth plate (Tian *et al.*, 2013). The TD is described as abnormal differentiation of GP chondrocytes, those normally lead to cartilage vascularization, mineralization and bone formation (Pines *et al.*, 1998; Shahzad *et al.*, 2014a). Etiologically, it is associated with several factors. However, most recent molecular study showed that the occurrence of TD is associated with inhibition of angiogenesis and genes related to bone vascularization and mineralization (Shahzad *et al.*, 2015; Nabi *et al.*, 2016).

Thiram (tetramethyl thiuram disulfide) is generally used as pesticide and fungicide in agriculture sector, but experimentally addition of thiram is used to induce TD in chickens (Rasaputra *et al.*, 2013). Thiram interferes with normal GP metabolism and development of chondrocytes which ultimately causes TD (Rath *et al.*, 2005). Deaths of chondrocytes in transitional zone appear to interrupt the endochondral bone development, which leads to the enlargment of GP and occurrence of TD (Rasaputra *et al.*, 2013).

[§]These authors contributed equally to this work.

Tetramethylpyrazine also known as ligustrazine (TMP; scientific name: *Ligusticum chuanxiong* Hort; Chinese name: chuanxiong) is one of the most important analgesic herb and active ingredients of traditional Chinese medicine. TMP has been identified with several molecular targeting properties, and demonstrates a broad therapeutic capacity such as scavenging oxygen free radicals (Li *et al.,* 2009; Fan *et al.,* 2011). It has antiapoptotic effect associated with down-regulating the expressions of Bcl-2 and Bcl-2 Associated X protein (bax) along with suppressing of caspase-3 (Chang *et al.*, 2007). It is widely used in China to treat neurovascular disorders, ischemic stroke, pulmonary hypertension and obstructive pulmonary diseases due to its effectiveness and low toxicity (Hintz and Ren, 2003; Liang *et al.*, 2005). In present study, we established an animal model to assess the effects of TMP for the treatment and control of TD in broiler chickens and its effect on liver antioxidant capabilities.

MATERIALS AND METHODS

Chemicals and reagents: Thiram was purchased from Shanghai Macklin Biochemical Co. Ltd. China, and Ligustrazine was purchased from the Deruifeng commercial company (Jinan, China). All other commercial reagents and kits were purchased from Jiancheng Biochem Company Nanjing, China.

Ethics approval: The animal experiments were conducted after the approval of institutional Animal Welfare and Research, Ethics Committee guideline of Huazhong Agricultural University (Wuhan, China).

Experiment design: One hundred and eighty, 1-day old Arbor Acres chickens (AACs), weighing 48±6g were purchased from commercial hatchery (Chengdu, China). Chicken were raised under standard hygienic conditions at temperature 93ºF, and humidity 60%. Initially, all the birds were offered standard feed and water ad libitum till day 3 post hatch. After that on day 4 post hatch, chickens were distributed in 2 groups; Control (n=60) and Thiram (n=120) group. Tibial dyschondroplasia was induced by adding thiram @ 50 mg per kg of feed to thiram group **(**Shahzad *et al.,* 2014a; Shahzad *et al.,* 2015; Iqbal *et al.*, 2016**)** while control group was offered normal diet. On day 7 post hatch, after induction of TD, thiram group was equally divided into two groups designated as TD group (n=60) and TMP treatment group (n=60). TMP group was treated with Tetramethylpyrazine @ 30 mg/kg/day (Fan *et al.,* 2011; Shin *et al.,* 2013; Zhang *et al.,* 2015) by intra-peritoneal route from day 7 to day 18.

Sample collection: Fifteen blood samples (n=15) were collected randomly from each group including control, TD and TMP treatment groups by cardiac puncture on day 7, 10, 14 and 18. Blood samples were centrifuged at 3000×g for 20min for separation of blood serum and stored at -70°C, until subsequent use and further analysis to determine the ALP activity, AST and ALT levels. The blood samples were collected before slaughtering from each group chickens for biochemical analysis. After the euthanizing the chicken, the tibial bone GP was measured, then some of the tibial bones were fixed in 4% paraformaldehyde for hematoxylin & eosin staining. The liver samples were also collected from each group on day 7, 10, 14 and 18. The liver samples were stored at -70°C for subsequent analysis of SOD, GSH-Px activity and MDA contents. Liver GSH-Px activity and SOD were assessed using assay kits (Nanjing Institute of Biological Engineering, Inc. Jiangsu, China) by following manufacturer's instructions and presented in U/mg protein (Unit per milligram of protein), while MDA contents were expressed in nmoles/g (nanomoles/gram) wet weight of liver tissue. AST, ALT and ALP activities were presented in U/L (unit per liter) according to previous studies (Nabi *et al.,* 2016).

Hematoxylin Eosin staining: The individual tibiotarsal bone samples were fixed immediately in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C, decalcified with 10% ethylenediamine tetra acetic acid, dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax. Sections were incised with 4µm thickness to prepare the slides. These paraffin-embedded sections were dewaxed in xylene and stained with H&E stain. Pathological changes in the tibial bones were observed under light microscope.

Statistical analysis: Data were examined with two-way ANOVA and student t-test to compare the differences between mean values of control and treatment groups. All the analyses were performed by using SPSS 19.0 software and data is presented as mean±SEM (standard error of means) and $P<0.05$ was considered statistically significant.

RESULTS

Effects of TMP on lameness, growth-plate size and morphology: The morphological examination revealed depressed and poor body condition of chickens in thiram-induced TD with reduced growth and lameness on day 7 to day 10 as compared to control group. After TMP treatment, the chickens started to regain their ability to walk and stand properly on day 10 (Fig. 1A). Tibia bone performance indicators are shown in Fig. 1B, the size of proximal tibial GP of thiram-induced TD chickens were markedly enlarged in the TD group. However, administration of TMP significantly reduced the TD score, size of GP, and the chickens started to walk properly. Histology of GP showed well-conserved columns with huge blood vessels in a proliferative and hypertrophic zone of GP. However, shrinked cells and irregular columns of chondrocytes were found in TD group. After TMP treatment, the width of hypertrophic region restored, angiogenesis was observed, and the growth plate become normal (Fig. 1C).

Biochemical criterion analysis of serum: A significant decrease in ALP activity, along with an increase in AST and ALT contents in the serum were observed during the entire experiment period in thiram induced TD chickens as compared to control group. However, TMP administration increased the ALP contents while decreased the AST and ALT contents significantly ($P<0.05$) near to control group (Fig. 2).

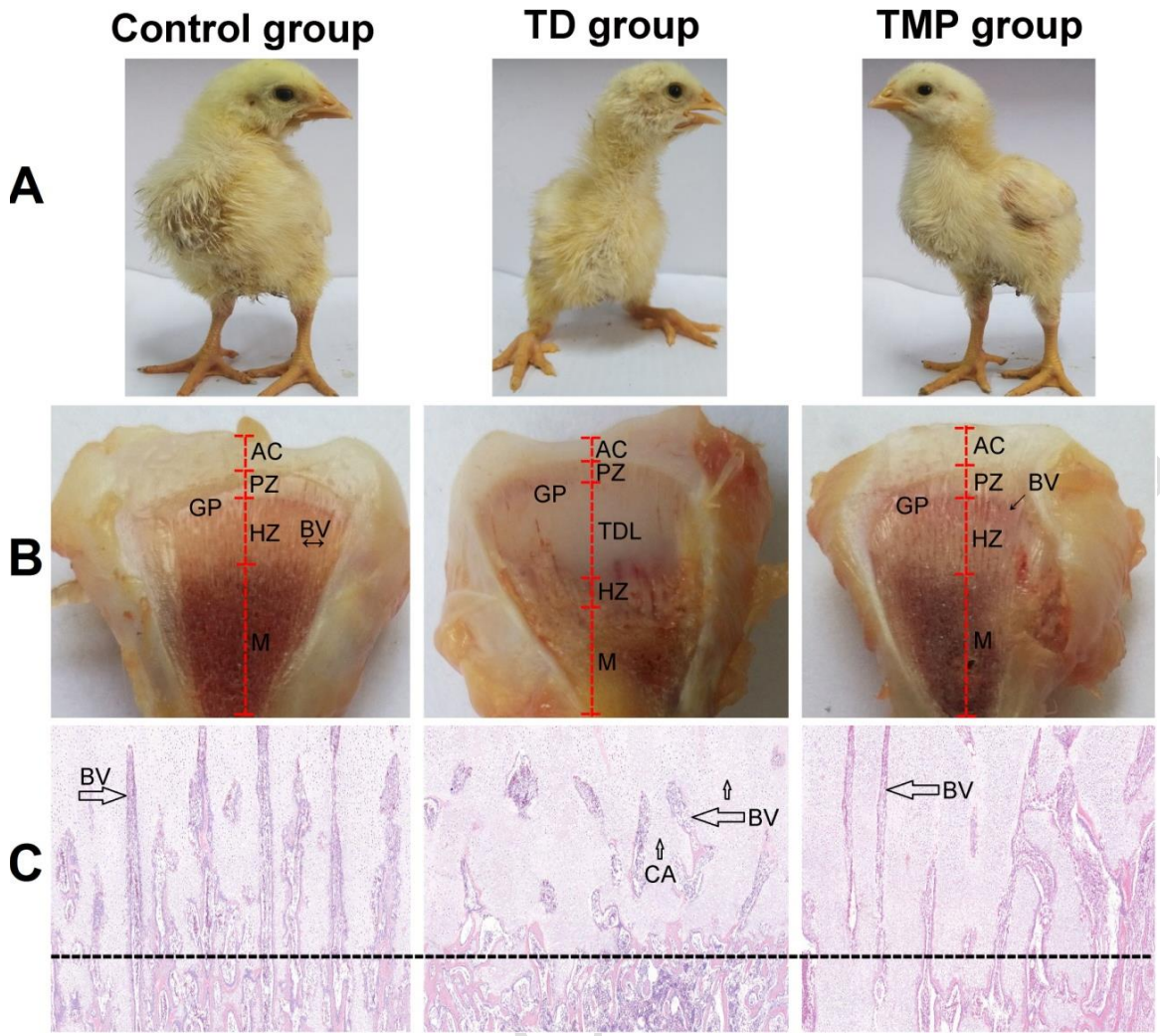

Fig. 1: Effects of TMP on thiram induced tibial dyschondroplasia chieckens. Lameness. AC=articular cartilage; PZ=proliferative zone; GP=growth plate; TDL=tibial dyschondroplasia lesion; HZ=hypertrophic zone; CA=cell apoptosis; M=metaphasis; BV=blood vessels. Stain: H&E; X400.

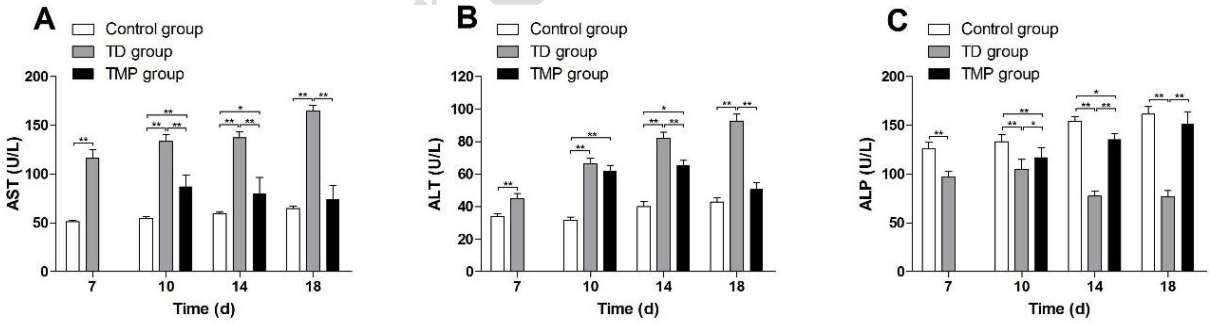

Fig. 2: Effect of thiram and TMP on serum parameters at various days. The data are presented in mean±SD. *P<0.05; **P<0.01.

Determination of oxidative stress in liver: The hepatic toxicity and oxidative stress in liver samples was measured to evaluate the effect of thiram and TMP treatments on liver damage in broiler chickens. The level of SOD, T-AOC and GSH-Px were significantly decreased (P<0.05), while the MDA level was increased in TD afflicted chickens as compared to control group. However, the intra peritoneal TMP administration caused an increase in SOD, T-AOC and GSH-Px activity with a decrease in MDA contents significantly (P<0.05) near to control group (Fig. 3).

DISCUSSION

Tibial dyschondroplasia is characterized by accumulated growth plate cartilage into metaphyseal region of the tibia, bone deformity and lameness, which affects fast growing broiler chickens worldwide. TD along with ascites and sudden death are regarded as the three nutritional and metabolic diseases that severely affect broiler industry (Julian, 1999). The occurrence of TD leads to huge economic loss to broiler industry worldwide (Edwards, 2000; Li and Bi, 2008).

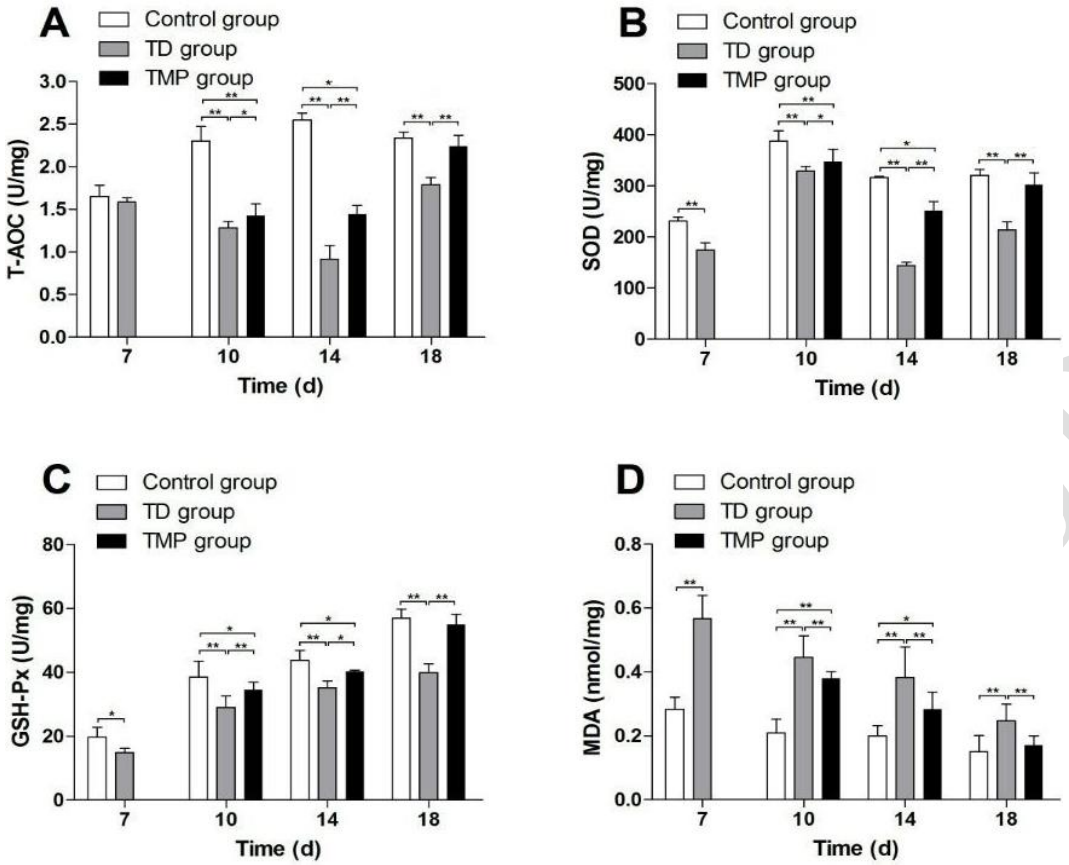

Fig. 3: Liver antioxidant activities analysis in broilers. The data are presented in mean±SD. *P<0.05; **P<0.01.

Normal broilers growth plate cartilage is circular arc with edge tidy, uniform thickness, and edge smooth (Tian *et al.,* 2013). TD occurred on proximal growth plate cartilage cell dysplasia, avascular, non-mineralized and opaque white cartilage wedge in growth plate, eventually led to the difficulties in movement and obstacles in standing (Li and Bi, 2008; Shahzad *et al.,* 2014a; Nabi *et al.,* 2016). Current study found that normal GP exhibits regular columns and cells surrounded by blood vessels in proliferative zone (PZ) and hypertrophic zone (HZ). While necrosis, reduced number of blood vessels (avascularized GP), and immature cartilage cells with cells arrange tightly in TD group. Whereas, vascularization, osteoclasts and osteoblasts scarce in proliferative zone, and apoptosis was also found in severe lesions tibia bone. After the treatment with TMP, TD was disappeared in the region of the cartilage lesions significantly and chicken regained their ability to walk properly.

To explore the pathogenesis of TD, thiram is widely used as an inducing factor to induce TD. However, it has been noticed that thiram has toxic effects on liver function (Rath *et al.,* 2004; Shahzad *et al.,* 2014b). Development of poultry tibial cartilage is coordinated with each other in a variety of enzyme, and most of the enzymes transform, mature and metabolize in the liver. It has been testified that thiram markedly prevent the GSH and SOD activity in human which leads to oxidative stress and imbalance (Marikovsky, 2002). This type of oxidative stress effects the lipid peroxidation of cell membrane and ultimately release of MDA as an end product (Perry *et al.,* 2010; Shahzad *et al*., 2014a). Dalvi and Deoras (1986) reported that thiram administration to rats result in significant increase level of AST which indicated the liver damages. AST and ALT contents in the serum are important indicator of liver injury. The content of ALP can indicate degree of cartilage cell differentiation and mineral substance, which is closely related to the cartilage reconstruction (Phull *et al.,* 2016). In current study, thiram significant increased the level of AST, which is an indicator to assess the liver function in chickens. In our study, antioxidant markers of the liver damages indicated that, the level of SOD, T-AOC and GSH-Px were significantly (P<0.05) decreased in TD group, while level of MDA was increased significantly (P<0.05) during the study period. However, TMP treatment caused an increase in the level of SOD, T-AOC and GSH-Px and decreased in MDA contents to normal level. TMP alleviated the damaging effects of thiram on the liver and corrected the oxidative imbalance. Toxicity and oxidative stress is considered mutual pathological mechanism due to several inflammatory or pathological situations in humans and animals (Li *et al.,* 2015). Several factors induce oxidative stress in liver, while liver is principal detoxifying organ that maintains metabolic homeostasis and directly impairs the production performance of chicken. Administration of antioxidants in case of liver damages and oxidative stress is a rational strategy to cure oxidative imbalance (Li *et al.,* 2015).

From the current study, it is concluded that Tetramethylpyrazine (traditional Chinese medicine) is a natural antioxidant which possess strong antioxidant and scavenge capability. It is supposed to be the basis of health benefits on avian tibial dyschondroplasia in broiler chickens.

Acknowledgments: The study was supported by National Key Research and Development Program of China (Project No. 2017YFD0502200), and the National Natural Science Foundation of China (31460682).

Authors contribution: KM and HZ made equal contributions in conducting experiments. JKL, HZ and KM provided the research idea. MKI, MUR, KL, SH, MS, and FN contributed reagents, materials, and analysis tools. KM, MI and HZ wrote the manuscript and handles the revision.

REFERENCES

Chang Y, Hsiao G, Chen SH, *et al.*, 2007. Tetramethylpyrazine suppresses HIF-1α, TNF-α, and activated caspase-3 expression in middle cerebral artery occlusion-induced brain ischemia in rats. Acta Pharmacol Sin 28:327-33.

Dalvi RR and Deoras DP, 1986. Metabolism of a dithiocarbamate fungicide thiram to carbon disulfide in the rat and its hepatotoxic implications. Acta Pharmacol Toxicol (Copenh) 58:38-42.

Edwards HM, 2000. Nutrition and skeletal problems in poultry. Poult Sci 79:1018-23.

Fan L, Wang K, Shi Z, *et al.,* 2011. Tetramethylpyrazine protects spinal cord and reduces inflammation in a rat model of spinal cord ischemia-reperfusion injury. J Vasc Surg 54:192-200.

Hintz KK and Ren J, 2003. Tetramethylpyrazine elicits disparate responses in cardiac contraction and intracellular Ca^{2+} transients in isolated adult rat ventricular myocytes. Vasc Pharmacol 40:213-7.

Iqbal MK, Liu J, Nabi F, *et al.,* 2016. Recovery of chicken growth plate by heat-shock protein 90 inhibitors epigallocatechin-3-gallate and apigenin in thiram-induced tibial dyschondroplasia. Avian Dis 60:773-8.

Julian RJ, 1999. Rapid growth problems: ascites and skeletal deformities in broilers. Poult Sci 77:1773-80.

Li J and Bi DR, 2008. Effects of high dietary vitamin A supplementation on tibial dyschondroplasia, skin pigmentation and growth performance in avian broilers. Res Vet Sci 84:409-12.

Li S, Tan HY, Wang N, *et al.,* 2015. The role of oxidative stress and antioxidants in liver diseases. Int J Mol Sci 16:26087-124.

Li XY, He JL, Liu HT, *et al.,* 2009. Tetramethylpyrazine suppresses interleukin-8 expression in LPS-stimulated human umbilical vein endothelial cell by blocking ERK, p38 and nulear factor-κB signaling pathways. J Ethnopharmacol 125:83-9.

Liang MJ, He LC and Yang GD, 2005. Screening analysis and in vitro vasodilatation of effective components from Ligusticum Chuanxiong. Life Sci 78:128-33.

Marikovsky M, 2002. Thiram inhibits angiogenesis and slows the development of experimental tumours in mice. Brit J Cancer 86:779-87.

Nabi F, Li K, Shahzad M, *et al.,* 2016. Gambogic acid inhibits Hsp90 expressions in thiram-induced tibial dyschondroplasia. Pak Vet J 36:224-6.

Orth MW and Cook ME, 1994. Avian tibial dyschondroplasia: a morphological and biochemical review of the growth plate lesion and its causes. Vet Pathol 31:403-4.

Perry JJP, Shin DS, Getzoff ED, *et al.,* 2010. The structural biochemistry of the superoxide dismutases. Biochim Biophys Acta, 1804:245-62.

Phull AR, Eo SH, Abbas Q, *et al.,* 2016. Applications of chondrocyte-based cartilage engineering: an overview. Biomed Res Int 2016:1879837.

Pines M, Knopov V, Genina O, *et al.,* 1998. Development of avian tibial dyschondroplasia: gene expression and protein synthesis. Calcified Tissue Int 63:521-7.

Rasaputra KS, Liyanage R, Lay JJ, *et al.,* 2013. Effect of thiram on avian growth plate chondrocytes in culture. J Toxicol Sci 38:93-101.

Rath NC, Huff WE, Balog JM, *et al.,* 2004. Comparative efficacy of different dithiocarbamates to induce tibial dyschondroplasia in poultry. Poult Sci 83:266-74.

Rath NC, Richards MP and Huff WE, 2005. Changes in the tibial growth plates of chickens with thiram-induced dyschondroplasia. J Comp Pathol 33:41-52.

Shahzad M, Gao J, Qin P, *et al.,* 2014a. Expression of genes encoding matrilin-3 and cyclin-I during the impairment and recovery of chicken growth plate in tibial dyschondroplasia. Avian Dis 58:468-73.

Shahzad M, Liu J, Gao J, *et al.,* 2014b. Hsp-90 inhibitor geldanamycin attenuates liver oxidative stress and toxicity in thiram-induced tibial dyschondroplasia. Pak Vet J 34:545-7.

Shahzad M, Liu J, Gao J, *et al.,* 2015. Differential expression of extracellular matrix metalloproteinase inducer (EMMPRIN/ CD147) in avian tibial dyschondroplasia. Avian Pathol 44:13-8.

Shin JW, Moon JY, Seong JW, *et al.,* 2013. Effects of tetramethylpyrazine on microglia activation in spinal cord compression injury of mice. Am J Chinese Med 41:1361-76.

Tian WX, Li J, Qin P, *et al.,* 2013. Screening of differentially expressed genes in the growth plate of broiler chickens with tibial dyschondroplasia by microarray analysis. BMC Genomics 14:276.

Zhang H, Song, Y, Li Z, *et al.,* 2015. Evaluation of ligustrazine on the prevention of experimentally induced abdominal adhesions in rats. Int J Surg 21:115-21.