Bulgarian Journal of Veterinary Medicine, 2026, **29**, No 2, 223–235
ISSN 1311-1477; DOI: 10.15547/bjvm.2025-0054

Original article

PUERARIN ATTENUATES THIRAM-INDUCED CYTOTOXICITY IN CULTURED CHICKEN GROWTH PLATE CHONDROCYTES: INSIGHTS INTO THE *IN VITRO* THERAPEUTIC EFFICACY BY MODULATING HIF-1A, TIMP-3, AND BCL-2 EXPRESSIONS

M. WAQAS[1,2 #], W. YAO[1,3 #], M. IQBAL[1,4], M. F. E. A. KULYAR[1], M. NAWAZ[2], Z. AHMED[2], A. JABBAR[2], O. ULLAH[2], F. A. KIANI[5] & J. LI[1]

[1]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, P.R. China; [2]Faculty of Veterinary and Animal Sciences, University of Poonch Rawalakot, Poonch, Azad Jammu & Kashmir, Pakistan; [3]College of Veterinary Medicine, Southwest University, Chongqing, P.R. China; [4]Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; [5]Department of Clinical Sciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

Summary

Waqas, M., W. Yao, M. Iqbal, M. F. E. A. Kulyar, M. Nawaz, Z. Ahmed, A. Jabbar, O. Ullah, F. A. Kiani & J. Li, 2026. Puerarin attenuates Thiram-induced cytotoxicity in cultured chicken growth plate chondrocytes: insights into the *in vitro* therapeutic efficacy by modulating HIF-1α, TIMP-3, and BCL-2 expressions. *Bulg. J. Vet. Med.,* **29**, No 2, 223–235.

Endochondral ossification is a crucial process in longitudinal bone growth, relying on chondrocytes proliferation, hypertrophy and cartilage matrix secretion. Disruption in this process, particularly due to cytotoxic agents like tetramethyl thiuram disulfide (Thiram), can impair skeletal development and induce apoptosis. The present research aimed to explore the *in vitro* protective role of Puerarin, a reputable bioactive isoflavone from Traditional Chinese Medicine on the growth plate (GP) chondrocyte's morphology and survival, as well as mRNA and protein expressions of hypoxia-inducible factor-1α (HIF-1α), tissue inhibitor of metalloproteinase-3 (TIMP-3), and B-cell lymphoma-2 (BCL-2) against Thiram-induced cytotoxicity in chicken growth plate chondrocytes. The chondrocytes from chicken tibial growth plates were obtained, cultured, refined, and divided into Control, Thiram and Puerarin groups. The chondrocytes in Thiram and Puerarin groups were subsequently treated with a sub-lethal dose of Thiram at 2.5 μg/mL to cause cytotoxicity, followed by an optimal dose of puerarin at 2.5 μg/mL to Puerarin group. Microscopy, RT-qPCR and Western blotting were used to investigate chondrocyte morphology and viability, and molecular expressions of key regulators i.e., HIF-1α, TIMP-3 and BCL-2. Thiram exposure resulted in diminished survival and drastic structural anomalies

[#] The authors contributed equally to this work.

of chondrocytes, upregulated HIF-1α, and downregulated TIMP-3 and BCL-2. Nonetheless, puerarin treatment efficiently counteracted these Thiram-induced structural and molecular alterations ($P<0.05$). These outcomes support puerarin being a protective agent and presents innovative therapeutic strategies against Thiram-induced cytotoxicity in chickens GP chondrocytes.

Key words: chickens, chondrocytes, genes, proteins, puerarin, Thiram

INTRODUCTION

Endochondral ossification governs longitudinal bone growth at the growth plate where chondrocyte activities including proliferation, hypertrophy, and the secretion of cartilage matrix plays a crucial role in chondrogenesis (Nilsson *et al.,* 2005). Chondrogenesis is a fundamental process in vertebrates (Lefebvre & Smits, 2005), involving cartilage formation followed by steady substitution with bone (Ma *et al.,* 2013). Any disruption in the sequential maturation of chondrocytes can halt endochondral ossification and lead to substantial apoptosis (Mehmood *et al.*, 2019).

Programmed cellular death/apoptosis is an exceptional characteristic of eukaryotes wherein the individual cell is killed to the benefit of the entire body (Huettenbrenner *et al.*, 2003). This process is commanded by BCL-2, which controls apoptosis, homeostatic and immune signaling (Martinou & Youle, 2011; Banjara *et al.,* 2020). HIF-1α controls the angiogenesis (Shi & Fang, 2004), ensures cell survival in hypoxia (Bae *et al.,* 2006), and is capable of transforming the phenotypes of cells responsible for immunity (Fagundes *et al.,* 2024). TIMP-3 orders angiogenesis (Abu El-Asrar *et al.,* 2022), and remodels the bone (Shen *et al.,* 2010).

Thiram, a pesticide which is extensively used by the agriculture sector, has greatly contaminated the environment because of its residual properties (Sankowska *et al.,* 2017; Kim *et al.,* 2024). The toxicity linked to residual Thiram in the environment has gravely affected livestock and human life (Martins *et al.,* 2018) and has been reported to lower the body's immunity and cause kidney failure and RBCs downfall (Chen *et al.,* 2024); and to be a major contributor to skeletal ailments in broilers (Iqbal *et al.,* 2024).

Based on archaeological evidence, the practice of Traditional Chinese medicine (TCM) in healthcare is more than 5000 years old (Pan *et al.,* 2014). It is an organized healthcare approach to cure illnesses and augment health and well-being (Matos *et al.,* 2021). Puerarin, an exceptional TCM, is an important isoflavone compound (Fig. 1) isolated from *Radix Pueraria* (Gegan) (Zhang *et al.,* 2006), a dried root of *Puerarin lobata* (Willd.) Ohwi (Wang *et al.,* 2022). Earlier research validates that puerarin provides protection against hepatic dysfunction, apoptosis and neuronal damage (Wei *et al.,* 2014; Liu *et al.,* 2023) and enhances the immunity and angiogenesis (Chauhan *et al.,* 2024; Geng *et al.,* 2024). Additionally, puerarin has been reported as having excellent *in vivo* remedial activity against avian tibial dyschondroplasia (Waqas *et al.,* 2020). Since Thiram is cytotoxic *in vitro* (Rath *et al.,* 2011; Cereser *et al.,* 2001) and causes the death of chondrocytes (Rasaputra *et al.,* 2013), we assumed that puerarin may prove to be effective in *in vitro* chicken model. Consequently, the present research was envisioned to explore the *in vitro* therapeutic effect of puerarin on the morphology and survival of GP chondrocytes,

and the mRNA and protein expressions of HIF-1α, TIMP-3 and BCL-2 against Thiram-triggered cytotoxicity of chondrocytes in chickens. The chemical structure of Puerarin is illustrated in Fig. 1.

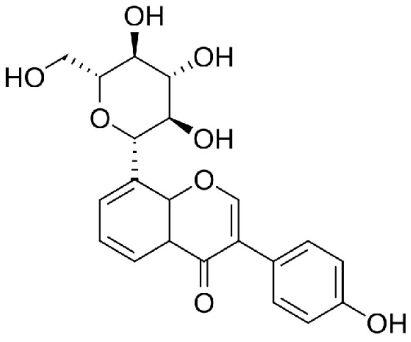

Fig. 1. Decoding puerarin: exploring the chemical architecture of a natural compound (https://en.wikipedia.org/wiki/Puerarin#/media/File:Puerarin.svg).

MATERIALS AND METHODS

Chickens, puerarin, reagents and antibodies

Fifty (n=50) day-old Arber Acre broiler chickens were obtained from Chia Tai Animal Husbandry Co. L.T.D., Jingzhou, China. Puerarin with 98% purity (Lot#: S02M9B54875) was procured from Shanghai Yuanye Biotechnology Co. L.T.D, China; Thiram (Lot#: C10036461) from Shanghai Macklin Biochemical Co. Ltd., China; Dulbecco's Modified Eagle Medium (DMEM) (gibco Lot#8119234) from ThermoFisher Biochemical Products, Co., L.T.D, Beijing, China. Foetal Bovine Serum (FBS) (gibco Lot#1861242), penicillin and streptomycin (PenStrep, Lot#2097437), collagenase and hyaluronidase were purchased from Life Technologies Corporation, Scoresby, Victoria, Australia; phosphate buffer saline (PBS) (HyClone™, Lot# AE27429263) from G.E Healthcare Life Sciences, Hyclone laboratories, South Logan, Utah, USA. Meilunbio® FG Super sensitive enhanced chemiluminescence (ECL) (MA0186) was manufactured by Dalian Meilun Biotechnology Co, L.T.D, Dalian, Liaoning, China; radio immunoprecipitation assay (RIPA) buffer (Lot#505644) – by ASPEN Biotechnology CO., LTD, Wuhan, China; bicinchoninic acid (BCA) protein detection kit – by Service Biotechnology, Wuhan, China. Trizol reagent was bought from Invitrogen, Carlsbad, CA, USA, and the first-strand cDNA synthesis kit from TransGen Biotech, Beijing, China. GenScript® (Nanjing, China) synthesised the primers for HIF-1α, TIMP-3, BCL-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The enhanced cell counting kit (CCK-8) (Lot#AI09295084), and rabbit polyclonal anti-HIF-1α (bs-20399R), anti-TIMP-3 (bs-0417R) and Anti-BCL-2 (bs-0032R) antibodies were acquired from Bioss Antibodies Inc. Woburn, Massachusetts, U.S.A.

Cell culture and digestion medium formulation

The cell culture medium contained 89% DMEM, 10% FBS, and 1% PenStrep. The digestion medium contained cell culture media, collagenase and hyaluronidase (Yao *et al.,* 2020).

Ethics approval, isolation, and culture of growth plate chondrocytes

The animal experiment adhered to the authorization and rules of the Ethics Committee of Huazhong Agricultural University, Wuhan, P.R. China. The isolation and culturing of chondrocytes followed established procedures outlined in prior reports (Yao *et al.,* 2020; Kulyar *et*

al., 2021; Ding *et al.,* 2021). Chickens aged 11 to 18 days were sacrificed; their growth plates were exposed, sliced into small pieces, and subsequently placed in digestive media and left to incubate overnight at 37 °C in a 5% CO_2 humidified shaking incubator to facilitate the release of chondrocytes. The resultant digestive media containing chondrocytes underwent filtration using a 70 µm cell strainer to eliminate debris. The filtrate was then centrifuged at 1000×g for 8 min. After discarding the supernatant, cell culture media was reintroduced and underwent another centrifugation at 800×g for 5 min to isolate chondrocytes. After two rounds of washing, the cells and culture media were seeded in six-well culture plates at 2×10^5 cells/mL per well. After adherent growth of first-generation chondrocytes covered the bottom of the culture plate to about 80%, 0.25% trypsin-EDTA was added to do sub-culturing.

Cells viability assay

The CCK-8 was used to determine the vitality of chondrocytes as previously outlined (Yao *et al.,* 2020). Various concentrations of puerarin, i.e., 1.5 µg/mL, 2.5 µg/mL, 5.5 µg/mL, 7.5 µg/mL, and 12.5 µg/mL (Wang *et al.,* 2013) were prepared by dissolving them in cell culture media and solutions were added to a 96 well plate. Afterward, the chondrocytes were seeded into each concentration and incubated for 6 hours. Henceforth, the CCK-8 was added into the wells and incubated for 2 hours followed by calculating the absorbance at 450 nm optical density using a Spectrostar Nano Microplate reader (BMG LABTECH GmbH, Germany). The concentration of puerarin at 2.5 µg/mL displayed the highest recorded viability, as determined by the provided equation (Yao *et al.,* 2020).

$$Cell\ viability = \frac{ODs \quad ODb}{ODc - ODb}\ x\ 100$$

where: ODb: blank controlled absorbance (culture medium and CCK-8); ODc: controlled absorbance (chondrocytes, culture medium and CCK-8); ODs: experimental wells absorbance (chondrocytes, culture medium, CCK-8, and puerarin) (Fig. 2).

Experimental framework

The GP chondrocytes seeded in six-well culture plates were washed with PBS, then divided into Control, Thiram, and Puer-

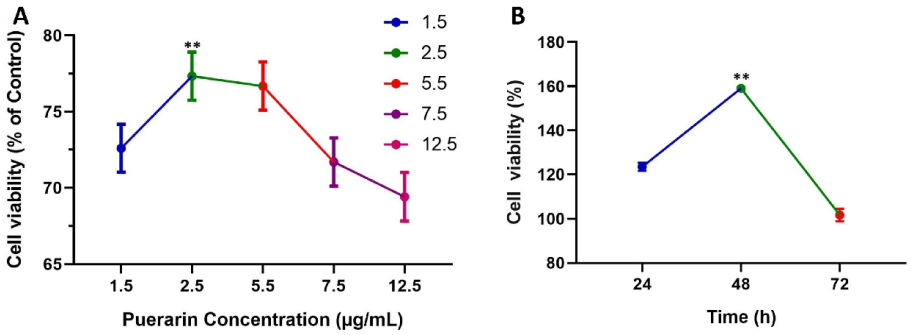

Fig. 2. The impact of puerarin concentrations of 1.5 µg/mL, 2.5 µg/mL, 5.5 µg/mL, 7.5 µg/mL, and 12.5 µg/mL on chondrocyte viability at 24, 48, 72 hours intervals, determined through the CCK-8 at an optical density of 450 nm. Data are presented as mean ± SD; ** P<0.0014.

Table 1. Primers employed in this investigation

Genes	Accession number	Primer sequences (5′-3′)	Product size (bp)
HIF-1α	Q16665	F: 5′-TGAGAGAAATGCTTACACACAG-3′ R: 5′-TGATGGGTGAGGAATTGGTTCAC-3′	263
TIMP3	NM_205487	F: 5′-CTCCAACTTCGGCCACTCA-3′ R: 5′- CAGGGATCTGTGGCATTGAT-3′	129
BCL-2	D_11382	F: 5′-GCAGGCAGCTTGAAAGAAAC-3′ R: 5′-GCTGGCCTTTCATGACTCTC-3′	180
GAPDH	NM_204305.1	F: 5′-GCCCAGAACATCATCCCA-3′ R: 5′-CGGCAGGTCAGGTCAACA-3′	137

arin groups and cultured in a standard culture media. Following this, Thiram and Puerarin groups chondrocytes were exposed to Thiram at a concentration of 2.5 µg/mL for 48 hours (Yao *et al.,* 2020). Next, the chondrocytes undertook rinsing and fresh media replacement followed by puerarin therapy at 2.5 µg/mL to Puerarin group chondrocytes for the next 48 hours. After the Thiram challenge and subsequent puerarin treatment, the structural alterations in chondrocytes across all groups were monitored microscopically.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction from GP chondrocytes of each group, i.e., Control, Thiram, and Puerarin, was done by Trizol method following the manufacturer's protocol as described earlier (Mehmood *et al.,* 2017). RNA integrity was confirmed via 1% agarose gel electrophoresis, and the concentration of RNA was assessed with Nanodrop 2000 analyzer (Thermo Scientific, Waltham, MA, USA). Following this, the RNA was transcribed reversibly to cDNA with first-strand cDNA kit with 1 µg of total RNA in a 20 µL reaction volume. Using particular primers (Table 1), the reactions were carried out with Step One-Plus™ RT-qPCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green (TransStart® Top Green qPCR SuperMix) with the following thermal profile: 95 ^{o}C for 30 s, followed by 40 cycles of 95 ^{o}C for 5 s, and 60 ^{o}C for 30 s. A melting curve analysis from 65 ^{o}C to 95 ^{o}C was performed to confirm amplification specificity (Wong & Medrano, 2005; Waqas *et al.,* 2019). Each reaction was done in triplicate, and the gene expression quantification was accomplished with the delta Ct ($2^{-\Delta\Delta Ct}$) technique using GAPDH as an internal control (Livak & Schmittgen, 2001).

Western blotting

After Thiram exposure and subsequent puerarin treatment, the protein extraction from chondrocyte lysate of all the groups was done by adding RIPA lysis buffer to the cell culture plate. The BCA protein detection kit was utilised to quantify the total protein concentration using a Spectrostar Nano Microplate reader, followed by protein denaturation by boiling at 100 ^{o}C and subsequent storage at −80 ^{o}C. The proteins underwent separation through 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes, exposed to 5% skimmed milk, and subjected to shaking for 1.5 hours at room temperature. Subsequently, membranes underwent five 5-

minute Tris-buffered Saline Tween (TBST) washes and were then incubated overnight at 4 °C with anti-HIF-1α, anti-TIMP-3, and anti-BCL-2 primary antibodies (1:1000). After washing, the PVDF membranes underwent incubation with secondary antibodies (1:3000) at room temperature for one hour. Henceforth, after washing with TBST four times, the images were taken with an imaging system using ECL reagent (Fusion Solo 7S. WL, France). Quantitative densitometry was performed using ImageJ (NIH, USA). Each target protein's expression was normalised against β-actin, and results were expressed as relative band intensities (target/β-actin). All values were statistically analysed across three biological replicates.

Statistical analysis

The GraphPad Prism v.8.0.2 (GraphPad Software Inc., San Diego, CA, USA) was employed to analyse the datasets through one-way analysis of variance (ANOVA) and Student's t-test, and to generate figures. Results are displayed as the mean ± standard deviation (mean ± SD), and statistical significance was documented at $P<0.05$.

RESULTS

Puerarin role in shaping chondrocyte's morphology

After thiram challenge, the chondrocytes in the Thiram and Puerarin groups got significantly reduced in number, displayed

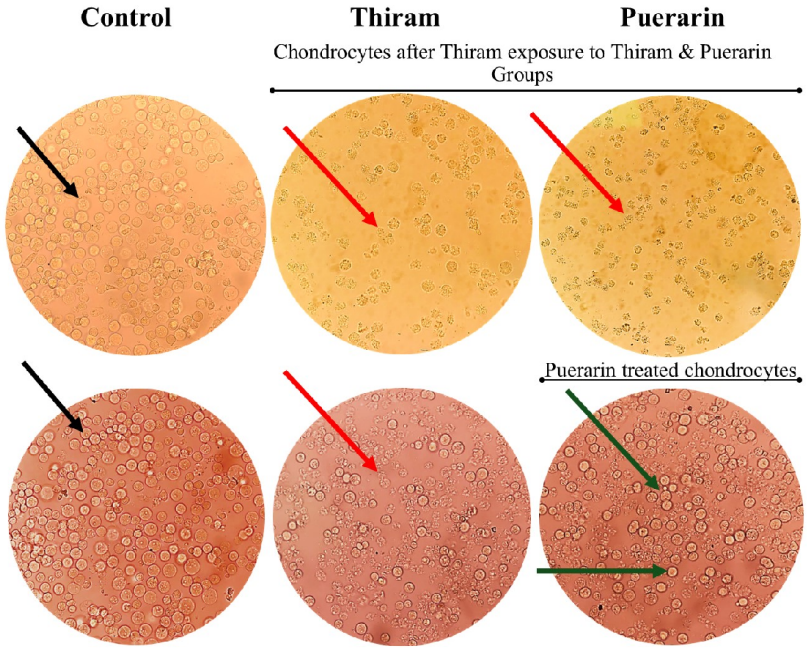

Fig. 3. Comparative analysis of chondrocyte morphology across various groups. Black arrows denote normal chondrocytes morphology in control group. Red arrows denote chondrocytes morphology in Thiram and Puerarin groups after Thiram exposure. Green arrows denote chondrocytes regaining their normal shape with puerarin treatment after Thiram exposure.

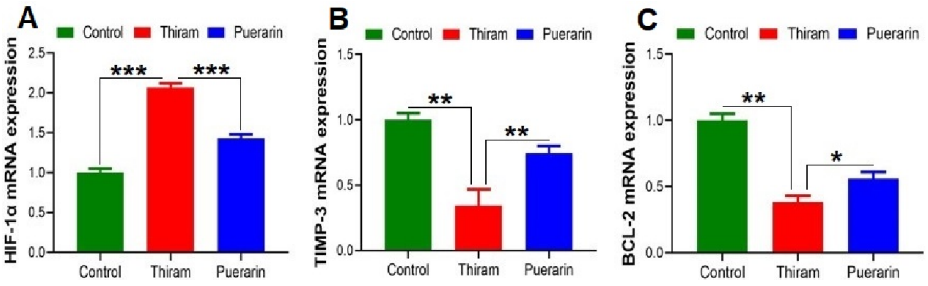

Fig. 4. Impact of puerarin on mRNA expressions of specific genes in growth plate chondrocytes. A, B, and C denote the levels of HIF-1, TIMP-3, and BCL-2 (* P<0.05, ** P<0.0011).

a peculiar shape with broken nuclei, scattered arrangement and increased apoptosis (Fig. 3). The chondrocytes in the Puerarin group previously exposed to thiram challenge were subsequently treated with puerarin. Following the treatment, chondrocytes nearly regained their normal shape and demonstrated superior adherence and external morphology compared to the Thiram group. Meanwhile, chondrocytes in the control group exhibited a uniform appearance, were plump, had a paving stone-like structure, and were closely arranged.

Puerarin-induced variations in mRNA expressions

Thiram challenge to the GP chondrocytes in the Thiram and Puerarin groups resulted in significant changes in expression levels of specific genes. HIF-1α showed upregulation, whereas TIMP-3 and BCL-2 exhibited downregulation compared to the control group. The chondrocytes in the Puerarin group that were earlier challenged with thiram and then treated with puerarin exhibited a noteworthy decrease in HIF-1α, coupled with an increase in TIMP-3, and BCL-2 expressions (P<0.05) (Fig. 4).

Puerarin-mediated shifts in protein expressions

After Thiram challenge, the Western blotting results depicted a significantly higher HIF-1α and lowered protein levels of TIMP-3 and BCL-2 in Thiram and Puerarin groups in comparison to control. Following exposure to thiram, the chondrocytes in Puerarin group underwent puerarin treatment which effectively restored the expression levels of these proteins by alleviating the Thiram toxicity damage inflicted on chondrocytes (P<0.05) (Fig. 5).

Correlation analysis

To further investigate the relationships among the expression levels of the genes studied, a Pearson correlation heatmap was generated using mRNA and protein data for HIF-1α, TIMP-3, and BCL-2. The analysis revealed strong intra-gene correlations between mRNA and corresponding protein levels for all the three genes (r>0.85, P<0.05). Inter-gene comparisons showed moderate correlations, suggesting coordinated regulation among these targets under puerarin treatment. This pattern supports a mechanistic alignment between transcriptional and translational responses contributing to the observed effects on chondrocyte function regulation (Fig. 6).

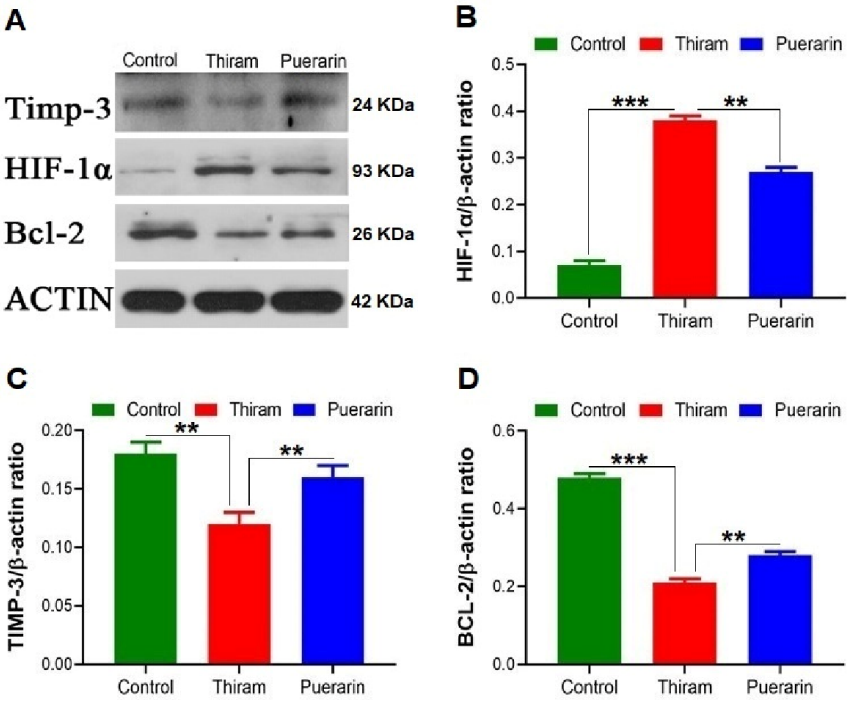

Fig. 5. Influence of puerarin on the protein levels of HIF-1α, TIMP-3, and BCL-2 detected by Western blotting technique (*P<0.05, **P<0.0018, ***P<0.0002).

DISCUSSION

Medicinal plants possess antioxidant (Men *et al.,* 2022), stress-relieving (Ajmal *et al.,* 2023), immunity enhancing properties (Abbas & Alkheraije, 2023) and protect against apoptosis (Zhu *et al.,* 2023). To evaluate the therapeutic protentional of puerarin, the chondrocytes were first exposed to Thiram followed by puerarin treatment. Thiram exposure exerted detrimental morphological effects on chondrocytes in the Thiram and Puerarin groups. Chondrocytes displayed a scattered arrangement with shattered nuclei, and their number greatly decreased which is consistent with previous research verifying Thiram toxicity on chondrocytes (Yao *et al.,* 2020; Kulyar *et al.,* 2021; Wu *et al.,* 2024). After puerarin treatment, the chondrocytes in the Puerarin group displayed significant improvement in shape, number, structure, and adherence compared to Thiram group. Our findings are congruent with former research that confirms puerarin enhances the survival of chondrocytes (Li *et al.,* 2021; Deng *et al.,* 2024).

HIF-1α controls chondrogenesis and angiogenesis (Huang *et al.,* 2017; Lefebvre & Smits, 2005). TIMP-3 modulates skeletal renovation and homeostasis (Chen *et al.,* 2019; Shen *et al.,* 2010) and protects chondrocyte from injuries (Liang *et al.,* 2016). BCL-2 impedes apoptosis and shields blood vasculature (Zaitoun *et*

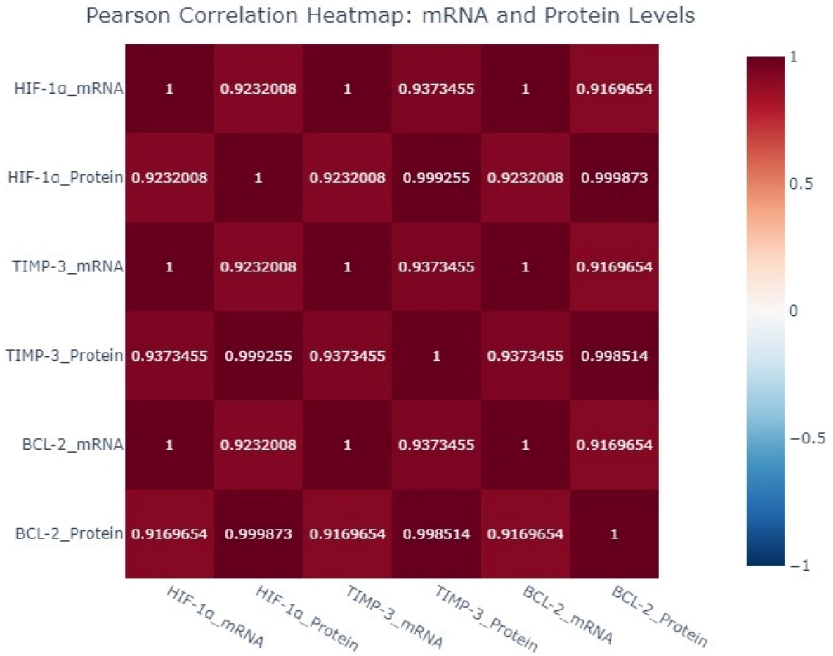

Fig. 6. Heatmap of Pearson correlation coefficients among mRNA and protein expression levels of HIF-1α, TIMP-3, and BCL-2 illustrating strong positive correlations between mRNA and corresponding protein levels within each gene, as well as moderate inter-gene associations indicating coordinated transcriptional and translational regulation under puerarin treatment.

al., 2019; Nagase *et al.,* 2009). In this research, after Thiram exposure to Thiram and Puerarin groups, the expressions of targeted genes and proteins were drastically altered, with HIF-1α being upregulated and at the same time, TIMP-3 and BCL-2 were downregulated, which confirms earlier research that Thiram results in raised HIF-1α expression (Zhou *et al.,* 2024; Ding *et al.,* 2021), and reduced BCL-2 (Zhang *et al.,* 2021; Chen *et al.,* 2024) and TIMP-3 expressions (Chen *et al.,* 2016; Waqas *et al.,* 2020). After treating chondrocytes with puerarin, the levels of targeted genes and proteins were considerably reinstated. Our research results support earlier studies that puerarin downregulates HIF-1α (Waqas *et al.,* 2020; Tang *et al.,* 2022) and enhances the TIMP-3 (Liang *et al.,* 2016; Waqas *et al.,* 2020) and BCL-2 expressions (Liu *et al.,* 2013; Waqas *et al.,* 2020; Cardona-Mendoza *et al.,* 2024).

CONCLUSION

Puerarin demonstrated its capacity to return chondrocytes to their typical morphology by successfully reducing the cytotoxic effects of Thiram. Additionally, puerarin reversed Thiram-induced damage by exercising regulatory control over specific genes and proteins. It increased TIMP-3 and BCL-2 at both mRNA and

protein levels and decreased HIF-1 suggesting that it may help survival of chondrocytes. All things considered, puerarin offers fresh viewpoints and shows considerable therapeutic promise as a substitute for shielding chondrocytes from Thiram-induced cytotoxicity.

ACKNOWLEDGEMENTS

The authors express special gratitude to Lihong Zhang and Ding Yanmei for technical support and help in completing this research successfully. This research received support from the National Natural Science Foundation of China (Grant No. 31460682) and the National Key Research and Development Program of China (Project No. 2017YFD0502 200). The funding entities did not shape the study's design, data collection, analysis, interpretation, manuscript preparation, or the decision to publish the findings.

REFERENCES

Abbas, A. & K. A. Alkheraije, 2023. Immunomodulatory effects of *Carica papaya* extract against experimentally induced coccidiosis in broiler chickens. *Pakistan Veterinary Journal,* **43**, 628–632.

Abu El-Asrar, A. M., A. Ahmad, M. I. Nawaz, M. M. Siddiquei, A. D. Zutter, L. Vanbrabant, P. W. Gikandi, G. Opdenakker & S. Struyf, 2022. Tissue inhibitor of metalloproteinase-3 ameliorates diabetes-induced retinal inflammation. *Frontiers in Physiology*, **12**, 807747.

Ajmal, A. S., Z. Hussain, M. M. Jalees, J. Shafi, S. Manzoor & A. U. Haq, 2023. Performance of broiler birds on feeding natural anti stressors in summer during heat stress. *Asian Journal of Agriculture & Biology*, **2**, 2022024.

Bae, M. K., S. H. Kim, J. W. Jeong, Y. M. Lee, H. S. Kim, S. R. Kim, I. Yun, S. K. Bae & K. W. Kim, 2006. Curcumin inhibits hypoxia-induced angiogenesis via down-regulation of HIF-1. *Oncology Reports*, **15**, 1557–1562.

Banjara, S., C. D. Suraweera, M .G. Hinds & M. Kvansakul, 2020. The Bcl-2 family: Ancient origins, conserved structures, and divergent mechanisms. Biomolecules, **10**, 128.

Cardona-Mendoza, A., A. Fonseca-Benitez, D. M. Buitrago, E. Coy-Barrera & S. J. Perdomo, 2024. Down-regulation of human papillomavirus E6 oncogene and antiproliferative effect of Schisandra chinensis and Pueraria lobata natural extracts on Hela cell line. *Journal of Ethnopharmacology*, **319**, 117225.

Cereser, C., S. Boget, P. Parvaz & A. Revol, 2001. An evaluation of thiram toxicity on cultured human skin fibroblasts. *Toxicology*, **162**, 89–101.

Chauhan, P., K. Wadhwa, R. Mishra, S. Gupta, F. Ahmad, M. Kamal, D. Iqbal, M. Alsaweed, M. V. Nuli, M. M. Abomughaid, A. G. Almutary, P. C. Mishra, S. K. Jha, S. Ojha, V. K. Nelson, A. Dargar, G. Singh & N. K. Jha, 2024. Investigating the potential therapeutic mechanisms of puerarin in neurological diseases. *Molecular Neurobiology*, **61**, 10747–10769.

Chen, J., G. Tellez & J. Escobar, 2016. Identification of biomarkers for footpad dermatitis development and wound healing. *Frontiers in Cellular and Infection Microbiology*, **6**, 26.

Chen, Y., A. Aiken, S. Saw, A. Weiss, H. Fang & R. Khokha, 2019. TIMP loss activates metalloproteinase-TNFα-DKK1 axis to compromise wnt signaling and bone mass. *Journal of Bone and Mineral Research*, **34**, 182–194.

Chen, Y., P. Tian, Y. Li, Z. Tang & H. Zhang, 2024. Thiram exposure: Disruption of the blood-testis barrier and altered apoptosis-autophagy dynamics in testicular cells via the Bcl-2/Bax and mTOR/Atg5/p62 pathways in mice. *Pesticide Biochemistry and Physiology*, **203**, 106010.

Deng, W., W. Zhang & Q. He, 2024. Study on the mechanism of puerarin against os-

teoarthritis from ferroptosis based on network pharmacology and bioinformatics. *Naunyn-schmiedeberg's Archives of Pharmacology*, **397**, 959–968.

Ding, Y., W. Yao, M. F. E. A. Kulyar, Q. Mo, H. Pan, Y. Zhang, B. Ma, Y. He, M. Zhang & J. Hong, 2021. Taurine is an effective therapy against thiram induced tibial dyschondroplasia via HIF-1α/VEGFA and β-catenin/GSK-3β pathways in broilers, *Ecotoxicology and Environmental Safety*, **228**, 112981.

Fagundes, R. R., A. Zaldumbide & C. T. Taylor. 2024. Role of hypoxia-inducible factor 1 in type 1 diabetes. *Trends in Pharmacological Sciences*, **45**, 798–810.

Geng, S., H. Zhang, Y. Zhang, L. Liu, S. Yu, X. Lan, Y. Gao, Z. Ling, Y. Zhang & X. Li, 2024. Puerarin hydrogel: Design and applications in biomedical engineering. *Journal of Drug Delivery Science and Technology,* **97**, 105802.

Huang, S. C., M. U. Rehman, Y. F. Lan, G. Qiu, H. Zhang, M. K. Iqbal, H. Q. Luo, K. Mehmood, L. H. Zhang & J. Li, 2017. Tibial dyschondroplasia is highly associated with suppression of tibial angiogenesis through regulating the HIF-1α/VEGF/VEGFR signaling pathway in chickens. *Scientific Reports*, **7**, 9089.

Huettenbrenner, S., S. Maier, C. Leisser, D. Polgar, S. Strasser, M. Grusch & G. Krupitza, 2003. The evolution of cell death programs as prerequisites of multicellularity. *Mutation Research*, **543**, 235–249.

Iqbal, M., M. Waqas, Q. Mo, F. A. Kiani, M. Shahzad, K. Mehmood, M. F. E. A. Kulyar, H. Qamar, Z. Zeng & S. Nawaz, 2024. Resveratrol alleviates thiram-induced tibial dyschondroplasia by regulating BMP-2, RUNX-2 and HIF-1α expressions in broiler chickens. *Toxin Reviews*, **44**, 33–47.

Kim, W., G. Kim, H. Park, K. Chai, J. Park & J. Park, 2024. Detecting and tracking thiram in leakage pathways using bioinspired nanograss with thuja fruit-like nanoparticles. *Sensors and Actuators B: Chemical,* **406**, 135405.

Kulyar, M. F. E. A., W. Yao, Y. Ding, H. Du, K. Li, L. Zhang, A. Li, P. Huachun, M. Waqas & K. Mehmood, 2021. Cluster of differentiation 147 (CD147) expression is linked with thiram induced chondrocyte's apoptosis via Bcl-2/Bax/Caspase-3 signalling in tibial growth plate under chlorogenic acid repercussion. *Ecotoxicology and Environmental Safety*, **213**, 112059.

Lefebvre, V. & P. Smits, 2005. Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Research Part C: Embryo Today: Reviews*, **75**, 200–212.

Li, G., H. Rao & W. Xu, 2021. Puerarin plays a protective role in chondrocytes by activating Beclin1-dependent autophagy. *Bioscience, Biotechnology, and Biochemistry*, **85**, 621–625.

Liang, Y., S. Chen, Y. Yang, C. Lan, G. Zhang, Z. Ji & H. Lin, 2016. Effect of Puerarin on TIMP3, MMP-9 expression and methylation in chondrocytes of rat osteoarthritis. *International Journal of Clinical and Experimental Medicine*, **9**, 17952–17957.

Liu, L., L. Liu, T. Bo, S. Li, Z. Zhu, R. Cui & D. Mao. 2013. Puerarin suppress apoptosis of human osteoblasts via ERK signaling pathway. *International Journal of Endocrinology*, **2013**, 786574.

Liu, X., R. Huang & J. Wan, 2023. Puerarin: A potential natural neuroprotective agent for neurological disorders. *Biomedicine & Pharmacotherapy*, **162**, 114581.

Livak, K. J. & T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, **25**, 402–408.

Ma, R. S., Z. L. Zhou, J. W. Luo, H. Zhang & J. F. Hou, 2013. The Ihh signal is essential for regulating proliferation and hypertrophy of cultured chicken chondrocytes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **166**, 117–122.

Martinou, J. C., & R. J. Youle, 2011. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Developmental Cell*, **21**, 92–101.

Martins, S. E., G. Fillmann, A. Lillicrap & K. V. Thomas, 2018. Ecotoxicity of organic and organo-metallic antifouling co-biocides and implications for environmental hazard and risk assessments in aquatic ecosystems. *Biofouling*, **34**, 34–52.

Matos, L. C., J. P. Machado, F. J. Monteiro & H. J. Greten, 2021. Understanding traditional Chinese medicine therapeutics: an overview of the basics and clinical applications. Healthcare, **9**, 257.

Mehmood, K., H. Zhang, M. K. Iqbal, M. U. Rehman, M. Shahzad, K. Li, S. Huang, F. Nabi, L. Zhang & J. Li, 2017. *In vitro* effect of apigenin and danshen in tibial dyschondroplasia through inhibition of heat-shock protein 90 and vascular endothelial growth factor expressions in avian growth plate cells. *Avian Diseases*, **61**, 372–377.

Mehmood, K., H. Zhang, X. Jiang, W. Yao, X. Tong, M. K. Iqbal, M. U. Rehman, M. Iqbal, M. Waqas & H. Qamar, 2019. Ligustrazine recovers thiram-induced tibial dyschondroplasia in chickens: Involvement of new molecules modulating integrin beta 3. *Ecotoxicology and Environmental Safety*, **168**, 205–211.

Men, T. T., N. D. H. Yen, L. T. K. Tu, T. N. Quy, N. T. K. Hue & D. T. Khang, 2022. Phytochemical constituents and antioxidant activity of some medicinal plants collected from the Mekong Delta, Vietnam. *Asian Journal of Agriculture & Biology*, **2022**, 202105230.

Nagase, Y., M. Iwasawa, T. Akiyama, Y. Kadono, M. Nakamura, Y. Oshima, T. Yasui, T. Matsumoto, J. Hirose & H. Nakamura. 2009. Anti-apoptotic molecule Bcl-2 regulates the differentiation, activation, and survival of both osteoblasts and osteoclasts. *Journal of Biological Chemistry*, **284**, 36659–36669.

Nilsson, O., R. Marino, F. D. Luca, M. Phillip & J. Baron, 2005. Endocrine regulation of the growth plate. *Hormone Research in Paediatrics,* **64**, 157–165.

Pan, S. Y., G. Litscher, S. H. Gao, S. F. Zhou, Z. L. Yu, H. Q. Chen, S. F Zhang, M. K. Tang, J. N. Sun & K. M. Ko. 2014. Historical perspective of traditional indigenous medical practices: the current renaissance and conservation of herbal resources. *Evidence-Based Complementary and Alternative Medicine,* **2014**, 525340.

Rasaputra, K. S., R. Liyanage, J. O. Lay Jr, M. F. Slavik & N.C. Rath, 2013. Effect of thiram on avian growth plate chondrocytes in culture. *The Journal of Toxicological Sciences*, **38**, 93–101.

Rath, N. C., K. S. Rasaputra, R. Liyanage, G. R. Huff & W. E. Huff, 2011. Dithiocarbamate toxicity – an appraisal. *Pesticides in the Modern World – Effects of Pesticides Exposure* **2011**, 323–340.

Sankowska, M., A. Gajek, M. Celinski & K. Satasinska, 2017. Determination of gaseous products of thermal degradation of thiram. *Journal of Thermal Analysis and Calorimetry*, **128**, 1639–1647.

Shen, Y., I. G. Winkler, V. Barbier, N. A. Sims, J. Hendy & J. P. Levesque, 2010. Tissue inhibitor of metalloproteinase-3 (TIMP-3) regulates hematopoiesis and bone formation in vivo. *PLoS One*, **5**, e13086.

Shi, Y. H. & W. G. Fang, 2004. Hypoxia-inducible factor-1 in tumour angiogenesis. *World Journal of Gastroenterology*, **10**, 1082.

Wei, S. Y., Y. Chen & X. Y. Xu, 2014. Progress on the pharmacological research of puerarin: A review. *Chinese Journal of Natural Medicines,* **12**, 407–414.

Tang, H., L. Kong, Y. Yang, J. Li & H. Zou, 2022. Puerarin suppresses hypoxia-induced vascular endothelial growth factor upregulation in human retinal pigmented epithelial cells by blocking JAK2/STAT3 pathway. *Bioengineered*, **13**, 11636–11645.

Wang, D., T. Bu, Y. Li, Y. He, F. Yang & L. Zou, 2022. Pharmacological activity, pharmacokinetics, and clinical research progress of puerarin. *Antioxidants*, **11**, 2121.

Wang, Y., Y. Ma, Y. Zheng, J. Song, X. Yang, C. Bi, D. Zhang & Q. Zhang, 2013. *In vitro* and *in vivo* anticancer activity of a novel puerarin nanosuspension against colon cancer, with high efficacy and low toxicity. *International Journal of Pharmaceutics,* **441**, 728–735.

Waqas, M., H. Qamar, J. Zhang, W. Yao, A. Li, Y. Wang, M. Iqbal, K. Mehmood, X. Jiang & J. Li, 2020. Puerarin enhance vascular proliferation and halt apoptosis in thiram-induced avian tibial dyschondroplasia by regulating HIF-1α, TIMP-3 and BCL-2 expressions. *Ecotoxicology and Environmental Safety*, **190**, 110126.

Waqas, M., Y. Wang, A. Li, H. Qamar, W. Yao, X. Tong, J. Zhang, M. Iqbal, K. Mehmood & J. Li, 2019. Osthole: A coumarin derivative assuage thiram-induced tibial dyschondroplasia by regulating BMP-2 and Runx-2 expressions in chickens. *Antioxidants*, **8**, 330.

Wong, M. L. & J. F. Medrano, 2005. Real-time PCR for mRNA quantification. *BioTechniques*, **39**, 75–85.

Wu, X., Y. Liu, Y. Li, Z. Tang, A. Li & H. Zhang, 2024. Molecular mechanism of thiram-induced abnormal chondrocyte proliferation via lncRNA MSTRG.74.1-BNIP3 axis. *Pesticide Biochemistry and Physiology*, **201**, 105847.

Yao, W., H. Zhang, M. F. E. A. Kulyar, Y. Ding, M. Waqas, K. Mehmood, M. Iqbal, H. Du, X. Jiang & J. Li, 2020. Effect of total flavonoids of *Rhizoma drynariae* in thiram induced cytotoxicity of chondrocyte via BMP-2/Runx2 and IHH/PTHrP expressions. *Ecotoxicology and Environmental Safety,* **206**, 111194.

Zaitoun, I. S., C. M. Wintheiser, N. Jamali, S. Wang, A. Suscha, S. R. Darjatmoko, K. Schleck, B. A. Hanna, V. Lindner, N. Sheibani & C. M. Sorenson, 2019. Bcl-2 expression in pericytes and astrocytes impacts vascular development and homeostasis. *Scientific Reports*, **9**, 9700.

Zhang, J., B. Luo, J. Liu, M. Waqas, M. F. E. A. Kulyar, K. Guo & J. Li, 2021. Chlorogenic acid inhibits apoptosis in thiram-induced tibial dyschondroplasia via intrinsic pathway. *Environmental Science and Pollution Research*, **28**, 68288– 68299.

Zhang, S., S. Chen, Y. Shen, D. Yang, X. Liu, A. C. Sun-chi & H. Xu, 2006. Puerarin induces angiogenesis in myocardium of rat with myocardial infarction. *Biological and Pharmaceutical Bulletin*, **29**, 945–950.

Zhou, S., C. Quan, Z. Zhang, S. Gong, S. Nawaz, Y. Zhang, M. F. E. A. Kulyar, Q. Mo & J. Li, 2024. Leucine improves thiram-induced tibial dyschondroplasia and gut microbiota dysbiosis in broilers. *Ecotoxicology and Environmental Safety*, **275**, 116260.

Zhu, S., Z. Ouyang, M. Kazim, Y. Qiu, S. Chen, X. Guan, J. Xiao, K. Mehmood, S. Wu, K. Liu, J. Pan, L. Hu, Y. Li, M. U. Saleem, J. Liao, Z. Tang & H. Zhang, 2023. Ameliorative effects of triptolide against autophagy and apoptosis in thiram induced tibial dyschondroplasia. *Pakistan Veterinary Journal*, **43**, 132–138.

Paper received 14.05.2025; accepted for publication 22.07.2025

Correspondence:

Muhammad Waqas
Faculty of Veterinary and Animal Sciences,
University of Poonch Rawalakot,
12350, Azad Jammu & Kashmir, Pakistan
contact: +92-345-9847205
e-mail: muhammadwaqas@upr.edu.pk