Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 06/11/2023;
Date Revised: 22/12/2023;
Date Published Online: 31/12/2023;

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How to Cite:
Raza R, Guangbin Z, Ahmad L, Maqbool B, Saleemi MK, Arshad MI, Khatoon A, Soomro H, Khan A and Gul ST (2023). Ameliorative effects of Vitamin E and Urtica dioica against thiamethoxam-induced teratogenicity in embryonated chicken eggs. Adv. Life Sci. 10(4): 577-584.

Keywords:
Teratogenic; Bird embryos; Vitamin E; *Urtica Dioica*

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Ameliorative effects of Vitamin E and *Urtica dioica* against thiamethoxam-induced teratogenicity in embryonated chicken eggs

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Abstract

Background: The purpose of this study was to explore the defensive effects of Vitamin E (Vit. E) and *Urtica dioica* (UD) in the mitigation of developmental anomalies induced by thiamethoxam (TMX) in chicken embryos.

Methods: For this purpose, a total of 140 fertile eggs were equally divided into seven experimental groups (A-G); Groups A and B were kept as negative and sham control, respectively. Group C was exposed to TMX while groups D and E were supplemented with Vit. E and *U. dioica*, respectively along with TMX. Group F received Vit. E and group G *U. dioica,* only. The eggs were examined on days 10th and 20th of incubation for the assessment of developmental flaws and musculoskeletal anomalies.

Results: The mortality rate was highest (40%) in group C (TMX) followed by groups D and E (20 and 15%), respectively. Developing embryos were exposed to TMX for retarded growth weight and crown-rump length (CRL) were significantly decreased as compared to the control group. The highest survival rate was recorded in negative control group A. The teratogenic defects recorded in this study include growth retardation, decreased crown-rump length, shortened beak, exencephaly, feather scantiness, and limb deformities. Morphometric analysis revealed improved growth by all parameters in Vit. E and *U. dioica* supplemented groups.

Conclusion: It was concluded that developmental defects are due to induced TMX, can be counteracted with Vit. E and *U. dioica* and have no phytochemicals negative effects.

Introduction

Pesticides include insecticides and rodenticides used to control some poultry pests; it can be necessary to apply chemicals directly to the flocks. The appropriate selection, handling, administration, and dumping of quality products within well-managed operations are required for the safe and efficient use of agrochemicals in various production systems. Four major problems have been reported due to the non-judicial use of insecticides at various levels including, health hazards (humans and animals), ecological damage, and productivity losses due to unexpected outcomes in terms of financial burdens on livestock and poultry farmers [1-4]. Potential environmental effects of the insecticides used in agriculture have been reported in literature like the death of wild birds and this number has increased in recent years. It might be related to the lavish use of systemic pesticides in recent years, particularly neonicotinoids and fipronil [5-8]. However, these describe the sustainability of toxic residues in poultry feed and then in meat and eggs obtained [9]. Other potential exposure routes at the hatchery phase for the fertilized eggs are routine cleaning, sanitation, and disinfection [10, 11]. These practices during the hygienic conditions applied during the setting for processing of fertilized eggs and raising chickens are much stricter than those in growth, reproduction, or laying operations. These eggs directly/indirectly come in contact with these chemicals and also absorb them through their pores [12-14].

Neonicotinoids (NEOs) were believed to have several potential adverse consequences on the ecosystem and people's health [15]. In the insects' central nervous systems (CNS), where nicotine acetylcholine receptors (nAChRs) are located, neonicotinoids chemicals operate as agonists of these receptors, paralyzing the insect muscles and causing their death [16]. As a systemic neonicotinoid insecticide, thiamethoxam is a cis-trans isomer. Therefore, TMX is used to exterminate different pests from crops. However, TXM-contaminated cereals cause many detrimental consequences in poultry birds. Chronic TMX exposure in poultry causes an extended hatching period, reduced egg output, and thinned eggshells [17]. Additionally, it also impaired the activities of the kidneys and liver. Therefore, increased levels of the enzymes (ALT and AST) as well as urea have been recorded. The laying hens' hematology, biochemistry, and production potential are all negatively squeezed by TMX residues [18]. TMX was found to be significantly teratogenic, causing liver abnormalities and developmental problems in chick embryos like growth retardation, head enlargement, limb abnormalities, scanty feathering, beak defects, ectopia visceral, decreased crown-rump length, decreased weight, and decreased head circumference [18].

While *Urtica dioica,* commonly known as stinging nettle, is a novel herbal product and has a lot of physiologically active ingredients in nettles. For instance, terpenoids, carotenoids, fatty acids, various essential amino acids, vitamins, tannins, minerals, carbohydrates, sterols, and polysaccharides are all abundant in the leaves [19, 20]. It has been demonstrated that *Urtica dioica* has antibacterial, antiviral, antioxidant, analgesic, anti-colitis, anti-inflammatory, anti-cancer, and anti-Alzheimer properties [21-25]. Nettle's antioxidant properties have been attributed to the presence of flavonoids, as well as its phenolic content. It has also been reported that nettle leaves have strong antioxidant properties and can control oxidative damage induced by the free radicles in cells. It protects tissues, proteins, lipids, and DNA from being damaged. *Urtica dioica* works through a free radicle scavenging effect and this ability ultimately results in hepatoprotective effects induced by free radicals production induced by the chemicals [26]. Vit. E has also been reported to counteract the adverse effects of neonicotinoids and ultimately reduces the oxidative stress on cells through the lipid peroxidation chain breaking and acting as a peroxyl radicle scavenger [27]. On the other hand, alpha-tocopherol is present in Vit. E inhibits the growth of free radicle production from membranes and prevents oxidative damage to it [28-31]. So, keeping in mind these potential antioxidant effects of Vit. E and *Urtica dioica* were evaluated to be an option to counteract the potential teratogenic effects of TMX in poultry where the residues have been reported in meat and eggs.

Methods

Before of the execution, study plan was approved by Graduate Studies and Research Board, and all the procedures were followed tailored by the Bioethics Committee, University of Agriculture, Faisalabad, Pakistan.

Chemicals

Thiamethoxam (TMX); chemical formula is $C_8H_{10}ClN_5O_3S$ [32]. Thiamethoxam was procured from the nearby market under the brand name "CONTEXT® WG25%".

Urtica dioica ethanol extract

Urtica dioica raw material (dried leaves) was purchased from a local herbal market and grinded in an electric mill. For three days, 50g of the *U. dioica* powder was allowed to macerate in 1:1 of 70% volume ethanol. The solution was then concentrated and filtered at 50°C in a rotary evaporator. This procedure was already

standardized [33] and was followed without any modification.

Vitamin E

Alpha-tocopherol acetate, also known as vitamin E, is a supplement that was purchased from the market in the form of 200 mg capsules as EVION®. Dose of Vit. E (0.1 mg/kg) was based on a previous study [34].

Experimental groups

A total of 140 embryonated eggs of the commercial broiler (ROSS) were procured from the local hatchery. Eggs were carefully cleaned in taped water for any dust particles or debris material. Candling of eggs was performed to confirm the viability of the embryos, tagging and slandered size eggs were marked the position of the air sac as they equally distributed in different groups. Eggs were examined for cracked, blood, or meat spot or any defective or unfertilized as the eggs were discarded. Then egg surfaces were cleaned with 70% alcohol solution for sanitization and were equally (20 eggs/group) divided into 7 groups A, B, C, D, E, F, and G groups (Table 1), respectively. Group A kept as a control. While Group B was sham control; given with normal saline for validation purposes. Group C eggs were injected with thiamethoxam which was administered into the allanto-amniotic sac (air sac). However, TMX and Vit. E were administrated to Group D and Group E eggs were given TMX and *Urtica dioica* extract (Figure 1). However, Groups F and G were inoculated with Vit. E and *Urtica dioica* extract on the third day of incubation, respectively. Two percent (2%) of eggs were examined for observing the different parameters. The eggs were instantly sealed with candle wax as shown in Figure 1.

Groups	Treatment Details
A (Control)	Untreated
B	Normal saline (20 µL/egg)
C	TMX (150 µg/egg)
D	TMX (150 µg/egg) + Vit. E (0.1 mg/kg)
E	TMX (150 µg/egg) + *Urtica dioica* (200 µL/egg)
F	Vit. E (0.1 mg/kg)
G	*Urtica dioica* (200 µL/egg)

Table 1: Experimental design using 3rd-day-old embryonated egg.

Post-treatment incubation

The eggs were incubated in an incubator at a temperature of (38±0.5°C), 60-80% humidity with standard protocols was given with narrow end down throughout the trail. Up to the 18th day turning was performed four times a day, as per the procedure described earlier [35]. To record teratogenic anomalies (if any) eggs were opened on the 20th day of incubation.

Parameters studied

Developmental defects were observed on the 10th and 20th day of incubation. Embryo weight, crown-rump length (CRL), beak size, head size, and shank length were measured and compared as per methods described earlier [36].

Statistical Analysis

One-way ANOVA was used to analyze the data gathered from the aforementioned parameters. Additionally, Tuckey's test (P≤0.05) was applied to compare means and determine significance. To know the difference between mortality within groups, Chi-square test was applied using Minitab Statistical Software [37].

Figure 1: *Urtica dioica ethanol extract* preparation and inoculation in 3-day-old embryonated eggs.

Results

Mortality rate and Survivability

Chi-Square test revealed significant (P<0.001) difference in mortality among various treatment groups (Table 2), being the highest (40%) in group C (TMX) followed by mortality in 20 and 10% in group D (TMX + Vit. E) and group E (TMX + *Urtica dioica*), respectively. Whereas no mortality (Table 2) was observed in groups A (Control - untreated), B (Sham Control), F (Vit. E) and G (*Urtica dioica*). The survival rate (Table 2) of embryo was 100% in Group A (Untreated-Control), B (Sham Control), F (Vit. E) and G (*Urtica dioica*), followed by 90% in Group E (TMX + *Urtica dioica*) and 80% in Group D (TMX + Vit. E). The lowest survival rate (60%) was noted in Group C (TMX).

Groups	Treatments with Dosage	Embryo Survivability		Mortality	
		No.	%	No.	%
A	Control (Untreated)	20	100	0	0
B	Normal saline (20µL/egg) Sham Control	20	100	0	0
C	TMX (150µg/egg)	12	60	8	40
D	TMX (150µg/egg) + Vit. E (0.1mg/kg)	16	80	4	20
E	TMX (150µg/egg) + *Urtica dioica* (200µL/egg)	18	90	2	10
F	Vit. E (0.1mg/kg)	20	100	0	0
G	*Urtica dioica* (200µL/egg)	20	100	0	0

Table 2: Mortality and survival rate of chicken embryos exposed to TMX and supplemented *Urtica Dioica.*

In each group, there were 20 chicken embryos. Mortality data analysis: Chi-Square Value = 21.646; df = 6; P-Value = 0.001.

Morphometric analysis of embryos

A morphometric examination between the treatment groups were recorded for the embryo weight and found significantly (P<0.05) lower in group C (TMX) fallowed by groups B, E and D, while it was significantly (P<0.05) higher in groups F and G supplemented with Vit. E and *Urtica dioica* along with group A (Table 3 and Table 4). The crown-rump length (CRL) was measured from head to bottom of the straightened chick embryo. CRL was significantly (P<0.05) downregulated in toxicity group C, while it was significantly upregulated in antioxidant treatment groups, supplemented with Vit. E and *Urtica dioica,* respectively, were showing results comparable to the control groups (Table 3 and Table 4).

The anterior-posterior head length was measured from the point of beak insertion to the occipital bone by using Vernier calipers. There was a significant reduction in head length in embryos exposed to TMX, while it was significantly improved in groups D and E. There was a non-significant reduction in relative head length as compared to control group. Beak length was measured from the beak tip to the point where it inserts into the skull Beak length was shortened non-significantly (P>0.05) in group C, while it was increased in groups D and E. Shank length and relative shank length were significantly reduced in embryos exposed to toxicity while both of these parameters were significantly (P<0.05) increased in groups that were offered with Vit. E and *Urtica dioica* as a treatment. In groups F and G were shown equal measurements as to control group (Table 3 and Table 4).

Teratogenic anomalies

Teratogenic parameters of embryos were evaluated. Some teratogenic effects were evaluated based on deviations from physical body parameters. The teratogenicity percentage was calculated by scorecard method, qualitatively. Group C (TMX) showed the overall the highest (30%) teratogenic rate (Table 5). While groups D (TMX + Vit. E) and E (TMX + UD) exhibited 15% and 10% teratogenicity, although groups F and G (Vit. E + UD) showed 0% teratogenicity between the treatment groups, respectively (Table 5).

Abnormalities observed in group C embryos included: rudimentary beak, agnathia, exencephaly, microcephaly, open eyes, visceral ectopia and micromelia (Table 4). While these teratogenic defects were significantly (P<0.05) pointed out in groups supplemented with Vit. E and *Urtica dioica*. These developmental imperfections are evident shown in gross pictures (Figures 2 and 3) at day 10th and 20th, respectively.

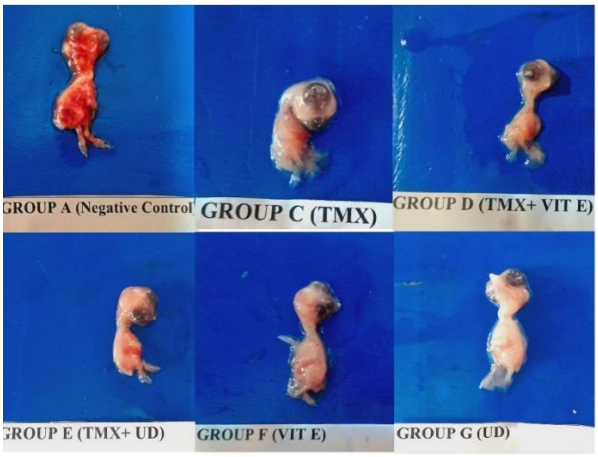

Figure 2: Photographs of chicken embryos on day 10th of incubation showing developmental imperfections, especially in TMX treated group.

Figure 3: Embryos on day 20th of incubation showing growth retardation, shortened upper beak and decreased CRL in TMX group while treatment groups are showing better growth of all parameters.

The results of the correlation analysis show that there are close links between such factors of growth in demand for FFs in the developed countries of the world as the "general nutritional status of the population", on the one hand, and the "disease incidence" (r_s = 0.652), as well as the "psycho-physiological profile of target consumer groups" (r_s = 0.612), on the other hand. At the same time, the correlation analysis also showed the relationship between the preferences of Kazakhstanis regarding the FFs and the factors in the development of the range structure of the FFs in Kazakhstan. Thus, close correlations were found between the preferences of Kazakhstan population regarding a variety of pro- and synbiotic dairy products and prebiotic food

Parameters	A	B	C	D	E	F	G
Body weight (g)	38.72±1.16^{a}	34.73±1.20^{b}	30.98±0.85^{c}	36.13±0.65^{b}	35.99±0.63^{b}	39.46±0.63^{a}	39.07±0.63^{a}
CR-length (cm)	7.82±0.28^{abc}	7.70±0.28^{abc}	6.49±0.24^{d}	6.67±0.22^{bcd}	6.65±0.21^{cd}	8.14±0.66^{a}	7.91±0.83^{ab}
Head length (cm)	2.51±0.14^{ab}	2.37±0.16^{abc}	2.03±0.15^{d}	2.20±0.08^{bcd}	2.20±0.08^{cd}	2.54±0.13^{a}	2.47±0.04^{abc}
Beak length (cm)	0.85±0.30^{a}	0.74±0.16^{a}	0.57±0.02^{a}	0.60±0.04^{a}	0.59±0.04^{a}	0.88±0.31^{a}	0.79±0.18^{a}
Shank length (cm)	3.05±0.13^{a}	2.85±0.12^{ab}	2.23±0.11^{c}	2.49±0.16^{c}	2.50±0.19^{bc}	3.16±0.05^{a}	3.11±0.10^{a}
Absolute liver weight (g)	0.37±0.02^{ab}	0.35±0.03^{bc}	0.31t±0.02^{c}	0.34±0.03^{bc}	0.35±0.04^{abc}	0.43±0.04^{a}	0.42±0.02^{ab}

Values (mean±SD) bearing different superscripts in a row differ significantly (P<0.05). Group A (Control-Untreated), Group B (Normal saline (20µL/egg)-Sham Control), Group C (TMX: 150µg/egg), Group D (TMX: 150µg/egg + Vit. E: 0.1mg/kg), Group E (TMX: 150µg/egg + *Urtica dioica*: 200µL/egg), Group F (Vit. E: 0.1mg/kg), and Group G (*Urtica dioica*: 200µL/egg).

Table 3: Absolute morphometric values of 20-day old embryos hatched from egg treated with thiamethoxam, Vitamin E and *Urtica dioica.*

Parameters	A	B	C	D	E	F	G
Relative head length	32.07±0.70^{a}	30.72±0.99^{a}	31.21±1.57^{a}	32.85±0.43^{a}	32.24±1.06^{a}	31.26±0.90^{a}	31.33±2.77^{a}
Relative beak	10.72±3.35^{a}	9.57±1.73^{a}	8.68±0.01^{a}	8.90±0.24^{a}	8.83±0.26^{a}	10.66±2.80^{a}	9.87±1.10^{a}
Relative shank length	39.03±0.55^{a}	37.02±0.47^{ab}	34.36±0.59^{b}	37.33±1.29^{a}	37.54±1.59^{a}	38.96±2.67^{a}	39.53±3.15^{a}
Relative liver weight	0.96±0.03^{a}	1.03±0.04^{a}	1.01±0.04^{a}	0.94±0.06^{a}	0.97±0.11^{a}	1.08±0.09^{a}	1.06±0.04^{a}

Values (mean±SD) bearing different superscripts in a row differ significantly (P<0.05). Group A (Control-Untreated), Group B (Normal saline (20µL/egg)-Sham Control), Group C (TMX: 150µg/egg), Group D (TMX: 150µg/egg + Vit. E: 0.1mg/kg), Group E (TMX: 150µg/egg + *Urtica dioica*: 200µL/egg), Group F (Vit. E: 0.1mg/kg), and Group G (*Urtica dioica*: 200µL/egg).

Table 4: Relative morphometric values of 20-day old embryos hatched from egg treated with thiamethoxam, Vitamin E and *Urtica dioica.*

Groups	Severity of Teratogenic Effect							Teratogenicity (%)
	Retarded Growth	Scanty Feathers	Limb deformation	Exencephaly	Enlarged head	Agnathia	Anophthalmia	
A	-	-	-	-	-	-	-	0
B	-	-	-	-	-	-	-	0
C	+++	++	+++	++++	++	++++	++	30
D	+	+	+	++	+	++	+	15
E	+	-	+	+	+	++	-	10
F	-	-	-	-	-	-	-	0
G	-	-	-	-	-	-	-	0

No degree = -; Mild = +, Moderate = ++; Severe = +++; Very Severe = ++++. Group A (Control-Untreated), Group B (Normal saline (20µL/egg)-Sham Control), Group C (TMX: 150µg/egg), Group D (TMX: 150µg/egg + Vit. E: 0.1mg/kg), Group E (TMX: 150µg/egg + *Urtica dioica*: 200µL/egg), Group F (Vit. E: 0.1mg/kg), and Group G (*Urtica dioica*: 200µL/egg).

Table 5: Qualitative analysis of teratogenic anomalies of embryos hatched from egg treated with treated with thiamethoxam, Vitamin E and *Urtica dioica.*

products from grain raw materials, on the one hand, and disease incidence, on the other hand (r_s = 0.711, r_s = 0.684, respectively).

Discussion

When an embryo develops, a series of decidedly organized biological processes take place. These developments start with extremely specific processes of metabolism as a result of fertilization, continue through the most delicate cell divisions and differentiations to produce body tissues, organs and their systems, and eventually a fully formed body/individual. Any physical or chemical change in environment can drastically alter the embryonic development by stunning the growth or functionally unable to perform their role.

Chemicals like pesticides are extensively used and have various teratogenic effects. For teratological research on zebrafish, mice, quail, and chicken embryos, clothianidin, imidacloprid, and thiacloprid were among the neonicotinoids that had previously been utilized [38, 39]. Moreover, a research has been conducted on mice and rabbits, thiamethoxam produced embryo toxicity and stunted development [40, 41]. Teratogenic effects of thiamethoxam (TMX) have been examined. The embryo weight of TMX (group C) was significantly lowered, however, significantly increased in groups (D and E) supplemented with Vit. E and *Urtica dioica* along with TMX, respectively. The crown-rump length was measured from head to bottom of the straightened chick embryo. CRL was significantly downregulated in toxicity group (C), while it was significantly upregulated in antioxidant treatment groups. Supplemented with Vit. E and *Urtica dioica,* respectively. In-ovo administration of TMX posed severe developmental defects like exencephaly and anophthalmia. In addition, scarce feathers, retracted yolk sac failure, limb abnormalities, and beak agnathia were found. The dose of thiamethoxam that causes these anomalies was 150µg/egg [18]. TMX causes teratogenic effects by inducing oxidative stress. This oxidative stress is responsible for DNA damage and cellular apoptosis. The intrinsic apoptotic cascade activated by TMX administration, which also caused

alterations in the transcripts of genes linked to apoptosis. An imbalance between pro- and antioxidant molecules is known as oxidative stress, and it causes global damage at organism level [42]. While by decreasing the activities of catalase, glutathione, and superoxide dismutase, as well as by boosting malondialdehyde (MDA) levels, TMX significantly increased oxidative stress in the exposed groups [43]. Moreover, physiological active components in *Urtica dioica* and Vit. E are polyphenols and flavonoids [21, 24, 27]. These dietary components act as antioxidants and may be crucial in reducing oxidative stress and the cellular, biochemical, and developmental abnormalities it causes [44].

Phytochemicals are chemical compounds produced by plants during the natural metabolic processes to resist bacteria, fungi, and virus infections [45, 46]. Among these medicinal plants, *Urtica dioica* has the potential to ameliorate toxic effects of various insecticides. This study intends to investigate *Urtica dioica* potential defenses against TMX-induced teratogenicity. The teratogenicity percentage was observed qualitatively, results showed that the Group C (TMX) have highest percentage teratogenic rate (30%). While in group D (TMX + Vit. E) and E (TMX + UD) revealed 15 and 10%, respectively. However, mortality rate was recording highest percent (40%) in group C (TMX) followed by group D and E, (20% and 15%), respectively. Developing embryos were exposed to TMX for retarded growth weight and CRL were significantly decreased as compared to control group. The observed teratogenic effects like growth retardation, agnathia, exencephaly, limb deformities and scanty feathers were not completely bottled-up by Vit. E and *Urtica dioica* supplementation, but these supplements significantly reduced these defects. These detrimental developmental effects are mostly avoided by Vit. E's protective role in preserving membrane integrity and improved intrinsic antioxidant capacity, which shields the cytoplasm and nuclear constituents from oxidative damage [27, 30, 47]. Decreased CRL, beak and shank length are attributed to TMX intoxication that results in lipid peroxidation and the generation of free radicals, damaging cells and causing oxidative stress. *Urtica dioica* works by the way of its free radicle scavenging effects. The antioxidant activity of nettle may be due to the presence of flavonoid chrysoeriol; also, its antioxidant activity may be attributed to its flavonoids and phenolic contents [22]. The administration of *U. dioica* extract and Vit. E significantly reduced the perturbations of these parameters comparatively. TMX causes degeneration of hepatocytes while *U. dioica* counteracts this defect by regenerating hepatic cells. *U. dioica* ethanol extract and vitamins E recovered hepatotoxicity induced by pesticides [48-50]. Vit. E and *U. dioica* have a particularly important and defensive effect against the deleterious effects of free radicals caused by thiamethoxam [21, 24, 27]. Both of these supplements have helped the developing embryo by providing a shield against reactive oxygen species [28, 30]. Hence, protected the embryos from cellular damage.

It is concluded that TMX exposure causes oxidative stress leading to developmental disruption and malformations including in-ovo mortality and decline in growth parameters. Supplementation of Vit. E and *U. dioica* have potential to counteract defects of TMX by the way of their antioxidant properties. Hence, Vit. E and *U. dioica* have rescuing effect against TMX exposure. In a broader aspect, it will reduce economic losses.

Source of Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions

Shafia Tehseen Gul and Ahrar Khan conceived the idea and tailored the research outlines. Rameen Raza, Latif Ahmad and Bakhtawar Maqbool carried out all laboratory work and collected the data under the supervision of Muhammad Imran Arshad and Aisha Khatoon. Data analysis and interpretation was carried out by Shafia Tehseen Gul, Muhammad Kashif Saleemi and Ahrar Khan. The manuscript was written by Shafia Tehseen Gul, edited by Zhang Guangbin and Hidayatullah Soomro. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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