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# Anti-cold Stress Effect of Aqueous Extract of Fruit Kernel of *Zanthoxylum armatum* DC. in Wistar Albino Rats

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## ABSTRACT

**Background:** Stress is a reflex reaction initiated by animals due to the inability to cope with a stressor, which may cause discomfort, illness, or sometimes death. The present study aimed to investigate the anti-cold stress potential of aqueous extract of *Zanthoxylum armatum* DC. (fruit kernel) in Wistar albino rats.

**Methods:** Rats were divided into five different groups (n=6). Cold stress was induced by exposing rats to cold environments during peak winter for 4 h/day for the 10-day experimental period. Groups 1 and 2 kept as healthy and stress-induced groups respectively. Group 3 received the *Withania somnifera* (100 mg/kg body weight) as a standard adaptogen. The Group 4 and 5 received *Z. armatum* at a dose rate of 250 and 500 mg/kg body weight, respectively. Haemato-biochemical, stress and hormonal profile of all the groups were analyzed.

**Result:** A significant hyperglycemia was observed in a stress-induced group compared to the healthy group. Elevated lipid peroxidase (LPO) and declined superoxide dismutase (SOD) levels were recorded in stress-induced group concerning the healthy group. Hormonal analysis exhibited significant hypercortisolism with significant reduction in thyroxine (T4) values in stress- induced group. However, levels of LPO, SOD and hormonal parameters were recovered towards normal levels in a group 5, receiving a extract of *Z. armatum* (500 mg/kg). The present study revealed the anti-cold stress potential of aqueous extract of *Z. armatum* (fruit kernel) at a dose rate of 500 mg per kg body weight in rats.


**Key words:** Anti-cold stress, Oxidative stress, Rats, *Zanthoxylum armatum* DC.

## INTRODUCTION

The livestock sector is an important subsidiary of agriculture for small and marginal farmers of India. It provides a fixed income source for the livelihood security of livestock owners of the country. Stress conditions like heat or cold has a detrimental effect on growth, reproduction, immune system and production performance in animals. It is a key factor for the deterioration of health and productivity in animals. Stress is a major factor for alterations in behavior, hormonal and oxidative status of animals. Stress is a reflex reaction initiated by animals due to the inability to cope with the stressor, which may cause several side effects from discomfort, illness, or death (Etim *et al*., 2013). Stress covers the behavioral and biological responses to various abiotic stressors like restraining, castration, dehorning, teeth clipping, malnutrition, weaning, overcrowding, exposure to adverse climatic conditions like chilly weather, heat, extensive workload and transportation, *etc*. (Kumar *et al*., 2012). The animal body tries to cope with stress by either long-term or short-term responses by involving the endocrine system and nervous system for sensory input respectively. When external stimuli or stressor exceeds the adaptation capacity of animals, the stress-related syndrome will occur in animals which may be responsible for immune suppression and illness. Stress can be mitigated through various managemental alterations or by using the traditional herbal medicine.

*Zanthoxylum armatum* DC. (Common name-Timur) is an evergreen, tiny, sub-deciduous and spiny tree commonly found in the valleys of the Himalayas, the north-east part of India, Nepal, Bhutan, Pakistan, Myanmar and Bangladesh. This plant belongs to the family *Rutaceae*. The *Zanthoxylum* species has been reported to have antioxidant, neuro-protective, anti-diabetic, gastro-protective, hepato-protective, lipid lowering, anti-hypertensive, cardio-protective, anti-inflamatory, analgesic and anti-spasmodic activities (Okagu *et al.,* 2021). This plant is referred to as magical due to its several benefits in the healthcare system. The fruits of *Z. armatum* are being used for the treatment of cold, cough, limb numbness, sore throat, high altitude sickness and vertigo. In Nepal, pickles made from fruits are found beneficial in cough and cold conditions. Extract of fruit powder has been found effective against gastrointestinal

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disorders (Barktullah *et al*., 2013). Natural phytocompounds like flavonoids, alkaloids, terpenoids, phenols, lignins and coumarins are present in *Z. armatum*. (Hertog and Wiersum, 2000). The supplementation of anti-stressor like nano selenium improved the production performance in commercial broilers during summer stress (Jamima *et al.,* 2020). The stress is a major factor for deterioration of health and decline in production performance of animals at temperate climate. Hence the study was planned to validate the anti-cold stress potential of *Z. armatum* using scientific methods. The Bhotiya tribe of the Himalayan region uses the decoction of *Z. armatum* (fruit kernel) as a remedy against cold stress in humans as well as in small ruminants, however scientific reports for anti-cold stress effect are not known yet either in humans or in animals. To elucidate the pharmacological or ethnobotanical relevance, the anti-cold stress effect of aqueous extract of fruit kernel of *Z. armatum* was studied in Wistar albino rats in the present study.

## MATERIALS AND METHODS

### Collection of plant material and extraction

*Zanthoxylum armatum* DC. (fruit kernel) was collected from Munsiyari region of Pithoragarh district of Uttarakhand. The plant material was authenticated by the botanist at GBPUAT, Pantnagar, Uttarakhand. The fruit kernel was cleaned and air dried and pulverized in electric grinder. Dried fruit kernel powder was subjected to extraction using water as solvent by columnar Soxhlet method at temperature of 40-41°C with standard protocol. The extracts were dried at 41°C temperature.

### Acute toxicity study

An acute toxicity study was carried out using a fixed dose method in a rat model for 14 days, as per AYUSH guidelines (CCRAS, 2018). Aqueous extract of *Z. armatum* has been fed orally to four groups (n=4; Group I, II, III and IV) at the dose rate of 1000 mg/kg body weight, 1500 mg/kg body weight, 2000 mg/kg body weight and 4000 mg/kg body weight, respectively. Rats were routinely checked for their body weight and the development of any clinical signs and symptoms. On day 15, rats were sacrificed under ether anaesthesia and blood samples obtained were subjected to routine hematological examinations such as Hemoglobin (Hb), packed cell volume (PCV), total erythrocytes count (TEC) and total leukocytes count (TLC).

### Evaluation of anti-cold stress activity of *Z. armatum* in rats

### Experimental design

Male albino Wistar rats (100-150 g) of either sex between 3-4 weeks age were obtained from Laboratory Animal Resource Section (LARS) of ICAR-Indian Veterinary Research Institute, Izatnagar (UP) and were kept in Laboratory Animal Production Section of, Indian Veterinary Research Institute, Mukteswar campus, Nainital (UK) with standard feeding and managemental conditions. Rats were divided randomly into five groups (n=6). The study was conducted during 2018-19 after due approval of Institute Animal Ethical Committee (IAEC) of ICAR-Indian Veterinary Research Institute, Mukteswar, Uttarakhand (Reg. No. 387/ GO/RBi/S/R/L/06/CPCSEA).


**Fig 1:** *Zanthoxylum armatum* DC. (a), fruit kernel (b) and its powder (c).

Anti-cold stress potential of *Z. armatum* (Local name-Timur, part used-fruit kernel, Fig 1) was evaluated in Wistar rat model. Cold stress was induced by exposing adult Wistar rats of either sex to cold environment during peak winter for 4 h/day during the 10 days experimental period (Debnath *et al*., 2011). Due to the prevailing natural temperate climatic conditions at the study area, the method was chosen for evaluation of anti-cold stress potential. Group 1 and 2 were kept as healthy and stress-induced (Positive control) groups, respectively. These groups did not receive any treatment. The Aswagandha *(Withania somnifera)* root powder was given to Group 3 at 100 mg per kg body weight dose rate and this served as the standard group in the experiment. Groups 4 and 5 received aqueous extract of Timur (fruit kernel) at the dose rate of 250 and 500 mg/kg body weight, respectively. Rats were sacrificed on the day 11 under ether anaesthesia.

### Parameters of study

### Hematology

Hemoglobin, PCV, RBC, WBC, differential leukocyte count was estimated as per standard methods.

### Serum marker enzymes

Parameters like serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), serum bilirubin, total cholesterol, blood urea nitrogen, serum creatinine and serum glucose were analysed by using standard methods.

### Oxidative stress indices

To assess oxidative stress, the level of LPO, SOD and Catalase (CAT) were determined in serum using commercial ELISA kits (Cayman chemical company, USA).

### Hormone analysis

Levels of stress hormones like Triidothyronine T3, Thyroxine T4 and Cortisol were determined using commercial available radioimmunoassay kits (Immunotech, Czech Republic).

### Statistical analysis

Data was subjected to statistical analysis using ANOVA and paired t-test and $P<0.05$ was considered statistically significant. Data was subjected for statistical analysis using ANOVA and paired t-test.

## RESULTS AND DISCUSSION

### Acute toxicity study

Rats of all four groups were alert and active throughout the trial period of 2 weeks. Fecal consistencies were found normal in all the groups. In group 2, there was a significant ($p<0.05$) increase in the body weight of rats on day 14 and 7 as compared to day 0 of the experiment (Table 1). In other groups changes in the body weights were non-significant throughout the experiment. All the haemato-biochemical parameters were within the normal range in all the groups (Table 2). The present study revealed that *Z. armatum* is

**Table 1:** Alteration of body weight (gm) of different rat groups during safety trial of *Zanthoxylum armatum* DC.

| Days | Body weight (gm) Group I | Body weight (gm) Group II | Body weight (gm) Group III | Body weight (gm) Group IV |
|---|---|---|---|---|
| 0th day | 183.33±14.530 | 140.00±11.547[a] | 140.00±10.000 | 153.33±3.333 |
| 7th day | 180.00±11.547 | 190.00±11.547[b] | 156.67±17.638 | 153.33±6.667 |
| 14th day | 196.67±8.819 | 206.67±6.667[b] | 176.67±17.638 | 180.00±15.275 |

Feeding of *Z. armatum* to the rats of Group I, II, III and IV was done @ 1000, 1500, 2000 and 4000 mg/kg b.wt. respectively; Significant at $p \leq 0.05$.

**Table 2:** Hematological parameters of different rat groups during safety trial of *Zanthoxylum armatum* DC.

| Group | Hb (gm/dl) | TLC ($\times 10^3$/µl) | TEC ($\times 10^6$/µl) | PCV (%) |
|---|---|---|---|---|
| I | 14.46±0.80[a] | 8450.00±275.379[ab] | 4.46±0.425[a] | 45.12±1.55[a] |
| II | 13.40±0.40[a] | 11983.33±1457.833[b] | 5.43±0.545[a] | 41.56±1.33[a] |
| III | 14.10±0.55[a] | 8000.00±763.763[a] | 4.40±0.450[a] | 43.90±1.83[a] |
| IV | 14.00±0.57[a] | 8466.67±409.607[ab] | 4.50±0.305[a] | 43.56±1.92[a] |

Feeding of *Z. armatum* to the rats of Group I, II, III and IV was done @ 1000, 1500, 2000 and 4000 mg/kg b.wt. respectively; Significant at $p \leq 0.05$.

**Table 3:** Alteration of body weight (gm) of rats under different groups of treatments.

| Days | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| 0th day | 118.67±5.84[a] | 158.33±7.265 | 166.67± 14.53 | 175.33±7.26 | 160.67±22.18 |
| 5th day | 179.00± 2.0[b] | 171.00±9.074 | 171.67± 14.51 | 189.00±7.02 | 154.00±15.5 |
| 10th day | 191.67±0.66[b] | 177.00±6.083 | 193.33± 13.64 | 200.00±4.04 | 178.00±16.0 |

Group I- Healthy; Group II- Stress-induced; Group III- Aswagandha @100 mg; Group IV- Timur @ 250 mg; Group V- Timur @ 500 mg dose rate; Significant at $p \leq 0.05$.

safe upto 4000 mg dose rate without any clinico-biochemical alterations.

**Evaluation of anti-cold stress activity**of ***Z. armatum*** **in rats**

Bodyweight data recorded a significant ($p<0.05$) increase in body weight of the healthy group on the day 5 (179.00± 2.0 gm) and 10 (191.67±0.6 gm) as compared to the day 0 (118.67±5.8 gm) day of the experiment. Other groups experienced only a non-significant increase in body weights on day 5 and 10 as compared to the initial day of the experiment (Table 3). The non-significant increase in body weights of all the groups except healthy control rats may be due to the effect of cold exposure on the food intake which might have resulted in retardation of growth rates.

Haemato-biochemical parameters evidenced significant ($p<0.05$) hyperglycemia in a stress-induced group (269.00 ± 30.76 mg/dl) in context to a healthy group (140.90±27.41 mg/dl); however, blood glucose levels were found within the normal range in a group 4 (125.00±2.00 mg/dl) receiving the Timur extract at a 250 mg per kg dose rate. Other hematological parameters were within the normal ranges (Table 4, 5). Stress of any kind and nature will up-regulate the transcription of PEPCK genes resulting in increased glucose formation (Wang, 2005; Andrew and Walker, 1999). The current study also evidenced the hyperglycemia in the stress-induced group which was restored well in rats receiving Timur extract. A proven theory explained that stress condition activates the hypothalamo-pituitary-adrenal (HPA) axis similarly to the sympathetic adreno-medullary axis. Our study evidenced that, supplementation of Timur extract prevented the hyperglycemia that arose from stress response in rats showing its possible effect as an anti-cold stress agent. A previous study reported the lowering of blood glucose levels by feeding the extract of *Z. armatum* leaves (Khan *et al*., 2018). Alam *et al.* (2018) also reported that feeding methanol extract of *Z. armatum* (fruit) caused a significant *($p<0.05$)* reduction in blood glucose concentrations in healthy mice. *Z. armatum* was found effective in diabetic mice with a significant reduction in blood glucose level from on 3rd to 15th day of the experimental period (Alam *et al*., 2018). Above reports are supporting the results of our study.

Oxidative stress indices witnessed a significant ($p<0.05$) increase in lipid peroxidase (LPO; 46.90±1.86 nmol/ml) and decrease in superoxide dismutase (SOD; 1.02±0.10 U/ml) levels in a stress-induced group concerning the healthy group. However, there were non-significant changes in the catalase level among all the groups (Table 6). However, levels of LPO and SOD were in a normal range (similar to healthy group) in rats receiving Timur at a 500 mg/Kg body weight dose rate. The level of oxygen demand can increase by various folds during exposure to cold in animals. Higher oxygen demand can result in higher production of reactive oxygen species ultimately causing oxidative stress at a greater level (Sahin and Gumuslu, 2004a; Terblanche *et al*., 2000). Summer stress in goats revealed the elevated MDA

**Table 4:** Haematological parameters of rats under different groups of treatments.

| Groups | Hb (gm/dl) | TEC ($\times 10^6$/µl) | TLC ($\times 10^3$/µl) | PCV (%) | L (%) | N (%) | M (%) | E (%) | B (%) |
|---|---|---|---|---|---|---|---|---|---|
| I | $14.60\pm0.30^a$ | $4.77\pm0.75^a$ | $8866.67\pm1521.5^a$ | $45.56\pm1.01^a$ | $25.00\pm3.78^a$ | $70.33\pm3.38^b$ | $8.00\pm1.52^c$ | $0.33\pm0.33^a$ | 0.00±0.00 |
| II | $15.00\pm0.30^a$ | $4.14\pm0.42^a$ | $8033.33\pm1887.1^a$ | $46.90\pm1.01^a$ | $19.00\pm3.00^a$ | $75.33\pm2.02^b$ | $5.67\pm1.76^{abc}$ | $0.00\pm.00^a$ | 0.00±0.00 |
| III | $15.46\pm0.89^a$ | $4.26\pm0.06^a$ | $8850.00\pm1803.0^a$ | $48.45\pm2.98^a$ | $20.33\pm2.02^a$ | $77.00\pm2.51^b$ | $2.67\pm0.88^{abc}$ | $0.00\pm0.00^a$ | 0.00±0.00 |
| IV | $14.86\pm0.24^a$ | $4.49\pm0.24^a$ | $6433.33\pm724.76^a$ | $46.45\pm0.80^a$ | $25.00\pm6.11^a$ | $65.33\pm6.43^a$ | $7.00\pm1.15^{bc}$ | $2.67\pm1.20^b$ | 0.00±0.00 |
| V | $14.20\pm1.20^a$ | $3.78\pm0.32^a$ | $8983.33\pm966.66^a$ | $44.23\pm4.00^a$ | $50.33\pm6.22^b$ | $48.33\pm5.45^a$ | $1.33\pm0.88^a$ | $0.00\pm0.00^a$ | 0.00±0.00 |

Hb- Hemoglobin; TEC- Total erythrocyte count; TLC- Total leucocytes count; PCV-Packed cell volume; L- Lymhphocytes; N- Neutrophils; M- Monocytes; E- Eosinophils; B- Basophils; Group I- Healthy; Group II- Stress-induced; Group III- Aswagandha @100 mg; Group IV- Timur @ 250 mg; Group V- Timur @ 500 mg dose rate; Significant at $p\leq0.05$.

and depleted SOD levels which were brought within the normal range due to supplementation of Fenugreek seeds and probiotics (Sahoo *et al.,* 2020). Parenteral administration of Selenium and vitamin E in buffalo during summer stress increased the feed intake with reduction in rectal temperature (Kumar *et al.,* 2019). Our study also reported the increased lipid peroxidation in plasma of cold exposed rats which were better prevented in treatment group receiving Timur at a 500 mg dose rate. The present study showed depleted SOD activity in the stress-induced group which may be due to the inactivation of the Superoxide dismutase enzyme during the scavenging of free radicals. Our results evidenced that, the group receiving *Z. armatum* prevented the excessive SOD utilization hence SOD levels were maintained within a normal range. Supplementation of Timur in rats prevented the consumption of SOD levels by compensating for the oxidative stress produced due to cold exposure. Several reports have shown the role of oxidative stress in cold-exposed individuals (Sahin and Gumuslu, 2004 b; Kaushik and Kaur, 2003; Terblanche *et al*., 2000). In present experiment, catalase values were found depleted in cold exposed rats (195.36±6.70 nmol/ml) whereas its level was better restored towards normal in a group 4 receiving the *Z. armatum* at a dose rate of 250 mg per kg body weight. Our phytochemical analysis of *Z. armatum* revealed a strong positive reaction for the presence of flavonoids, saponins and phytosterols in the aqueous extract of the fruit kernel. Flavonoid compounds may exhibit an additive effect on the enzymatic and nonenzymatic defense mechanism of the cells or organisms (Nijveldt *et al*., 2001). It can be directly involved in free radical scavenging activity also. The presence of flavonoids in *Z. armatum* extract may be responsible for the prevention of oxidative damage in cold-exposed rats.

The hormonal profile revealed that the level of cortisol (228.1±60.85 nM/L) was increased significantly ($p<0.05$) in a stress-induced group which was restored well in a group receiving Timur at a 500 mg dose rate (106.398±23.573 nM/L). Significant ($p<0.05$) reduction in thyroxine (T4; 23.32 ± 1.313 nM/L) level and insignificant reduction in triiodothyronine (T3; 1.054±0.138 nM/L) levels were observed in a stress-induced group as compared to a healthy group (Table 6). Cortisol is a major stress hormone that is negatively correlated to the time of exposure to the stressor (Miller *et al*., 2002). As discussed earlier, stress can regulate the HPA axis resulting in increased production of cortisol. Our study demonstrated the cold-induced stress response in rats which was diminished by supplementing the aqueous extract of *Z. armatum*. Several reports published in past have shown the largest interest in phytopharmaceuticals and plant bio-molecules which can modulate the hypothalamic-pituitary-adrenal (HPA) axis along with cholinergic, monoaminergic system and catecholamines (Sasaki *et al*., 2013; Xu *et al*., 2010). Our results showed that feeding of Timur extract lowered the cortisol levels in cold-exposed rats which demonstrate the efficacy of aqueous extract of *Z. armatum* against cold stress.It is evidenced that, like the HPA axis, the hypothalamic- pituitary–thyroid (HPT) axis is also activated during stress conditions (Kilburn *et al*., 2010). Earlier research by Helmreich *et al.* (2005, 2006) demonstrated a significant decrease in T3 and T4 levels in stress-induced adult rats. Current findings also evidenced

**Table 5:** Biochemical parameters of rats under different groups of treatments.

| Group | ALT (IU/ml) | AST (IU/ml) | Glucose (mg/dl) | Cholesterol (mg/dl) | Creatinine (mg/dl) | BUN (mg/dl) |
|---|---|---|---|---|---|---|
| I | 68.22±63.35 | 80.23±64.53 | 140.90±27.41$^{ab}$ | 54.50±3.304 | 2.75±0.47$^{ab}$ | 8.25±0.25$^{ab}$ |
| II | 71.50±12.79 | 83.00±62.06 | 269.00±30.765$^{b}$ | 49.75±5.603 | 1.00±0.00$^{a}$ | 13.25±2.496$^{ab}$ |
| III | 52.25±17.19 | 33.25±3.351 | 218.50±23.22$^{ab}$ | 48.25±3.11 | 2.75±0.479$^{ab}$ | 9.00±0.00$^{ab}$ |
| IV | 50.00±0.0 | 42.50±14.50 | 125.00±2.0$^{a}$ | 49.50±1.50 | 3.00±0.00$^{aa}$ | 23.50±18.5$^{b}$ |
| V | 97.25±30.19 | 95.50±40.53 | 240.50±2.39$^{ab}$ | 55.00±3.18 | 3.00±0.57$^{ab}$ | 6.50±1.04$^{a}$ |

ALT- Alanine aminotransferase; AST- Aspartate aminotransferase; BUN- Blood Urea Nitrogen; Group I- Healthy; Group II- Stress-induced; Group III- Aswagandha @100 mg; Group IV- Timur @ 250 mg; Group V- Timur @ 500 mg dose rate; Significant at $p\leq0.05$.

**Table 6:** Oxidative stress and hormonal parameters of different groups.

| Group | LPO (nmol/ml) | SOD (U/ml) | CAT (nmol/ml/min) | T3 (nM/L) | T4 (nM/L) | Cortisol (nM/L) |
|---|---|---|---|---|---|---|
| I | 9.23±1.00$^{a}$ | 2.43±.24$^{b}$ | 283.55±89.77$^{ab}$ | 1.335±0.172$^{ab}$ | 28.235±3.323$^{c}$ | 56.878±22.475$^{a}$ |
| II | 46.90±1.86$^{d}$ | 1.02±.10$^{a}$ | 195.36±6.70$^{a}$ | 1.054±0.138$^{a}$ | 23.32±1.313$^{ab}$ | 228.1±60.856$^{c}$ |
| III | 27.88±2.24$^{bc}$ | 1.31±.28$^{ab}$ | 275.79±56.44$^{ab}$ | 1.312±0.217$^{a}$ | 26.268±0.738$^{b}$ | 97.005±19.006$^{ab}$ |
| IV | 34.79±1.91$^{cd}$ | 1.36±.28$^{ab}$ | 338.10±16.26$^{ab}$ | 0.961±0.054$^{a}$ | 21.56±0.078$^{a}$ | 211.08±34.097$^{c}$ |
| V | 10.96±3.46$^{a}$ | 1.84±.15$^{ab}$ | 214.84±22.47$^{ab}$ | 1.633±0.393$^{b}$ | 30.15±0.150$^{c}$ | 106.398±23.573$^{b}$ |

LPO-Lipid peroxidase; SOD- Superoxide dismutase; CAT- Catalase; T3-Tri-iodothyronine; T4- Tetra-iodothyronine; Group I- Healthy; Group II- Stress-induced; Group III- Aswagandha @100 mg; Group IV- Timur @ 250 mg; Group V- Timur @ 500 mg dose rate; Significant at $p\leq0.05$.

the lower levels of T3 and T4 in cold stress. Mild stress may or may not increase the thyroid hormone levels (Turakulov *et al*., 1994) but severe stress can cause a decrease in thyroid hormone levels significantly (Kilburn *et al*., 2010). Helmreich and Tylee (2011) revealed the reduction in thyroid hormone levels in stress- induced rats. The *Z. armatum* may be responsible for increasing the non-specific resistance in the rats exposed to the colder climate. Due to this, the hormonal changes might have restored towards normal in *Z. armatum* treatment group. This report also supports the anti-cold stress effect of *Z. armatum*.

## CONCLUSION

The current study investigated the anti-cold stress potential of *Zanthoxylum armatum* DC. in rats. Aqueous extract of *Z. armatum* (fruit kernel) at 500 mg dose rate exhibited the better anti-cold stress activity in a rat model. Supplementation of *Z. armatum* maintained the normal activity of oxidative stress markers and stress hormones in rats. Fruit kernel of *Z. armatum* is reported to have higher concentrations of compounds like flavonoids, saponins and phytosterols, which may be responsible for the prevention of oxidative stress and enhancement of non-specific resistance in rats. Further investigations can be designed for the identification of active bio-molecules responsible for the anti-stress activity of Timur.

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**Conflict of interest:** None.

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