# Immune-modulation in arsenic exposed buffaloes by *Terminalia arjuna (l.)*

S.K. Dash[1]*, B. Panda[2], S. Nayyar[3] and R. Jindal[3]

[1]Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Birsa Agricultural University, Ranchi, Jharkhand, [2]Department of Plant Breeding and Genetics, College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, [3]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India.
*Corresponding author e-mail: drsubratvbc@gmail.com

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## Abstract

The study investigated the effect of *Terminalia arjuna* bark powder on circulatory immune complexes and total immunoglobulins level in female Murrah buffaloes ingesting arsenic contaminated water and fodder in Ludhiana district, Punjab, India. Buffaloes were divided into three groups; Group-I (Control) includes ten healthy buffaloes from arsenic uncontaminated area, Group-II (Arsenic exposed) includes ten buffaloes from arsenic contaminated area and Group-III (Treatment) includes ten buffaloes from the arsenic contaminated area orally administered with *Terminalia arjuna* bark powder @ 42 mg/kg.b.w. by mixing with approximately 40grams of jaggery once daily for 30 days. Arsenic concentration was significantly ($p<0.05$) higher in drinking water, fodder and buffalo blood samples collected from the arsenic contaminated area as compared to uncontaminated area (control area). Buffaloes reared in the arsenic contaminated area showed significantly ($p<0.05$) elevated circulatory immune complexes with decreased level of plasma total immunoglobulins compared to the control. Buffaloes of the treatment group exhibited significant ($p<0.05$) decrease in the level of circulatory immune complexes; and increase in the total immunoglobulin concentration to the level comparable to that of control.

**Keywords:** Arsenic contaminated water; Immune complexes; Immunoglobulins; Buffalo; *Terminalia arjuna*.

# Introduction

Heavy metal toxicity is a matter of concern for livestock in general and large ruminant in particular across the world (Baeva et al., 2021; Khamikoeva et al, 2021). Arsenic is a naturally occurring metalloid, widely distributed in the environment. Arsenic has been detected in different concentrations in fodder, crops and water in many geographical locations (Nath *et al*. 2021). Contamination of drinking water through natural release of arsenic from aquifer rocks is the primary source of exposure in livestock. Apart from this, green fodder grown in arsenic contaminated area is another source of arsenic exposure to livestock (Kavil *et al*. 2020). Arsenic is very toxic and can cause several health problems in animals and human depending on their concentration and duration of exposure. It can cause dispigmentation, cancers of skin, liver and lungs, hematological, circulatory, reproductive, neurological gastrointestinal and immunological pathologies. However, the contribution of many environmental chemicals including arsenic in the etiology of these diseases is not established till now (Alam *et al.* 2021). Exposure to chemical compounds can affect the immunity either by causing hypersentivity and autoimmunity, in which the immune cells fails to differentiate between self and non-self, causing damaging effect on various organs of the body; or by causing immunosuppression in which activity and response of immune system is decreased. Immune complexes or antigen-antibody complexes are persistently formed in the body. Antigen and antibody interacts with each other by non-covalent forces. Presence of circulatory immune complexes (CICs) is an indicator of normal immune response as it is a part of humoral immunity. In case the immune response is effective, phagocytes eliminate the circulatory immune complexes from blood circulation (Mood *et al.* 2021). However, during abnormal immune response, circulatory immune complexes can't be eliminated from the circulation and can cause a variety of systemic disorders. CICs are detected in case of arsenic and other metal exposure, allergy, autoimmune diseases, rheumatoid arthritis, bacterial, viral, parasitic and cancerous conditions in humans (Dangleben *et al.* 2013, Tang *et al*. 2022). Circulatory immune complexes can also be used as indicators of environmental metal contamination including arsenic contamination in bovines (Ramanaviciene *et al.* 2004). Immune complex formation and deregulated immune function due to chronic arsenic exposure in human and animals has also been reported by various research workers (Selgrade 2007, Vahter 2008). However, effect of arsenic exposure on circulating level of immune complexes and immunoglobulins status in buffalo remains to be investigated.

Traditionally ethnomedicines are widely used in India for treatment of various disorders due to their easy accessibility, low cost and fewer side effects. In recent years there is increasing demand of plant derived therapeutics. Medicinal herbs have also proven immunoprotective and immunomodulatory potential. Powders and extracts prepared from them are widely used in the treatment of immunological disorders. The bark of *Terminalia arjuna*, a deciduous tree of the Combretaceae family, has been reported in ancient Indian medicinal literature as well as in current literature for having beneficial effects on various chemical mediated immune disorders (Ramesh and Palaniappan 2023). *Terminalia arjuna* bark contains many active constituents, such as tannins, triterpinoid saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids, ellagic acid, gallic acid, oligomeric proanthocyanidines, phytosterols, calcium, magnesium, zinc and copper. Metal chelating and immunomodulatory properties of *Terminalia arjuna* dried bark powder have been established in human and laboratory animals (Kanthe *et al.* 2021).

The present study investigated the status of immunoglobulins and circulating immune complexes in female murrah buffaloes environmentally exposed to arsenic through contaminated drinking water and fodder. The immune-modulatory effect of *Terminalia arjuna* bark powder in environmentally arsenic exposed buffaloes was also investigated.

# Materials and methods

**Ethical approval**

Experimental protocols of this study have been approved (No. 638/VBC/IAEC) by the Institutional Animal Ethical Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

**Study area**

The Koom Kalan village of Ludhiana district, Punjab was selected through survey, where the drinking water arsenic concentration was above the maximum permissible limit (0.01 µg/ml) described by the WHO (2005). It was observed that the village Koom Kalan of Ludhiana which is the starting point of Buddha Nullah is highly contaminated with arsenic. Buddha Nullah, an old seasonal water stream originates from village Koom Kalan (latitude $30^0$92'07.21'N, longitude $76^0$06'55.30'E) of Ludhiana, runs parallel and drains into the Sutlej river (latitude $30^0$97'49.74'N, longitude $75^0$62'69.02'E). During its course in the Ludhiana district, the water of the stream is polluted by the untreated industrial effluents from various industries located in and around Ludhiana. A study conducted on water samples of villages in and around Buddha nullah reported the presence of high concentration of arsenic in Koom Kalan village (Kaur *et al.* 2021). The Dairy farms located in Guru Angad

Dev Veterinary and Animal Sciences University, Ludhiana without any arsenic contamination problem was taken as control area. Tube well water, fodder and buffalo blood samples were collected from the dairy farms located in arsenic contaminated and control areas. The adult female murrah buffaloes (3-5 years of age) used in the study were maintained in organized dairy farms and provided with standard diet and ad libitum water.

**Study methodology**

**Collection of samples**

**Water**

Tube well water samples (100 ml) were collected in duplicate from the dairy farms located in arsenic contaminated (n=20) and control area (n=10) of Ludhiana. These tube wells were used for irrigation and supply of drinking water to the buffaloes. Water samples were collected in polypropylene bottles prewashed with nitric acid (1ml/L). Collected water samples were preserved in concentrated hydrochloric acid (4ml/L) and stored at 4$^{o}$C in refrigerator till estimation of total arsenic.

**Fodder**

Fodder samples used for feeding of buffaloes were collected in duplicate from the arsenic contaminated (n=20) and control area (n=10). These samples were washed with 2% hydrochloric acid and distilled water to remove all the impurities and dust particles. After removing the extra water with blotting paper, samples were cut into pieces, packed into petridishes, and kept in an oven for drying. The dried samples were grinded and passed through a sieve of 2 mm size and then kept at room temperature till estimation of arsenic.

**Blood**

Jugular vein blood (3ml)was collected from the buffaloes of the arsenic contaminated (n=20) and control area (n=10) using a sterile syringe and needle and transferred to a heparinized vial. Blood was transported from the field in an ice box. The whole blood samples were used for quantitation of total arsenic.

**Laboratory analysis of Samples**

Laboratory analysis of different samples has been carried out in the Department of Veterinary Physiology and Biochemistry, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

**Determination of arsenic concentration**

**Water**

Water samples were directly analyzed for the estimation of total arsenic.

**Fodder and Blood**

Fodder samples (1g) and blood samples (3ml) were digested using 15 ml of tri-acid mixture ($HNO_3$, $H_2SO_4$, and $HClO_4$ in 10:4:1 ratio) until a transparent solution was obtained. After cooling, the digested sample was filtered using Whatman No. 42 filter paper and final volume of filtrate was made to 10 ml with distilled water.

**Estimation of arsenic**

Concentration of arsenic in the water, fodder and blood samples was estimated using atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, Germany) with a flow injection hydride generation system (FIAS 100). All the determinations were performed in duplicate.

**Procurement of *Terminalia arjuna* bark powder (TABP)**

*Terminalia arjuna* bark powder used for the treatment of buffaloes was obtained from Nature Natural Ayurvedic Life Care company. Quality of *Terminalia arjuna* bark powder was maintained by Nature Natural Ayurvedic Life Care; which was congruent to the Ayurvedic Pharmacopoeia of India (API) as well as to Indian Pharmacopoeia (Indian Pharmacopoeia Commission 2014). The dose of *Terminalia arjuna* bark powder (42mg/kg b. w.) was decided as per the API.

**Experimental Buffaloes**

A total of thirty adult female Murrah buffaloes (same animals used above for determination of blood arsenic level) were divided into following groups:

Control group (n=10): Clinically healthy buffaloes selected from the uncontaminated area without any treatment (with blood arsenic level within the normal limit (0-0.05ppm).

Arsenic exposed/ Exposure control group (n=10): Buffaloes exposed to arsenic through intake of contaminated water and fodder selected from the arsenic contaminated area (with blood arsenic level above the normal limit of 0-0.05ppm).

Treatment group (n=10): Arsenic exposed buffaloes orally administered with *Terminalia arjuna* bark powder @ 42 mg/kg.b.w. by mixing with approximately 40 grams of jaggery once daily for 30 days.

All the buffaloes were approximately of same age and body weight. *Terminalia arjuna* bark powder was mixed with the above mentioned quantity of jaggery and smeared on the tongue of buffaloes. The treatment schedule did not cause any change in feed and water intake pattern of animals. Three blood samples (3ml) were collected during the supplementation period i.e. on day 0, 15 and 30 and one blood sample was collected after the withdrawal of supplementation i.e. on 45$^{th}$ day from buffaloes of the treatment group. Single blood samples were

collected from animals of the control and arsenic exposed groups. The blood samples were centrifuged at 2500 rpm for 10 minutes and plasma was separated.

**Circulatory immune complexes**

Circulatory immune complexes in plasma were determined by polyethylene glycol (PEG) precipitation method (Ramanaviciene *et al.* 2004). 2.7 ml of 0.1 M borate buffer (pH 8.4) and 0.3 ml of plasma were poured into a centrifuge tube and mixed. Then 4.5 ml of 5% PEG solution was added and the mixture was incubated for 2 hours at 4ºC followed by centrifugation at 600 g for 20 minutes. Supernatant was decanted and 3.5 ml of 3% PEG solution was poured over it, stored for 18 hours at 4ºC and centrifuged under the same conditions. The supernatant was discarded, sediment was mixed by beating, 0.3 ml of distilled water and 2.7 ml 0.1N sodium hydroxide was poured over it. Absorbance was determined at 280 nm using UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, Germany). The CIC concentration (mg/mL) was calculated using bovine IgG calibration curve. All determinations were performed in duplicate.

**Total immunoglobulins**

To 0.1 ml plasma, added 0.1 ml of 90% saturated ammonium sulphate (SAS) to get an overall concentration of 45% saturation. At this concentration of ammonium sulphate, immunoglobulins were precipitated after refrigeration at 4°C overnight followed by centrifugation at 6000 rpm for 15 minutes. The precipitate was washed twice with 45% SAS and pellet was dissolved in 0.1 ml phosphate buffer saline (pH - 7.4). 0.01ml of this solution was used to estimate the total immunoglobulins (Oser, 1965) . To 0.01ml of solution (diluted 10 times), added 5ml of alkaline copper solution, mixed well and allowed to stand for 10min. Then added 0.5mL of Folin-Ciocalteau reagent and incubated at room temperature in the dark for 30min. The volume was made up to 1 ml with distilled water. Standard was prepared by taking 20-200µg BSA/ml. Absorbance was recorded at 520 nm against blank using UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, Germany). All determinations were performed in duplicate.

**Statistical analysis**

Data was analyzed using Statistical package for Social Sciences (SPSS) software. Multiple comparisons of data were carried out by one way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test. Additional statistical comparisons between means of different groups were carried out using independent t-tests. Correlation was determined by Karl Pearson's coefficient of correlation.

# Results and Discussion

**Arsenic concentration in tube well water, fodder and buffalo blood**

In the current study, arsenic concentration was reported to be significantly ($p<0.05$) increased in tube well water, fodder and buffalo blood samples (Table 1) collected from arsenic contaminated area of Ludhiana district compared to the control area. Significant positive relationship ($r = 0.516$, $p<0.05$) was observed between arsenic concentration in tube well water and fodder samples from the arsenic contaminated area. Arsenic levels in the tube well water and fodder samples from arsenic contaminated area exhibited positive correlation ($p<0.05$) with blood arsenic level of untreated buffaloes reared in these areas ($r = 0.821$ and 0.672 respectively) (Table 2).

Environmental arsenic contamination problem is increasing year by year in different parts of the world. Large number of animals in different parts of globe especially of India is reported to be affected with arsenic toxicity through drinking water and consumption of contaminated fodder (Nath *et al.* 2021). Ludhiana, the industrial hub of Punjab, India is currently at risk of arsenic toxicity (Virk 2020). In Punjab, dairy animals are mostly supplied with the tube well water for drinking. Frequent use of underground water has lowered the water level; contaminating it with arsenic containing salts and minerals. Increased concentrations of sulfate, phosphate and hydroxyl ions along with an increased pH (>8.0) in the ground water aquifers of Punjab are responsible for the increased release of arsenic (Virk 2020). Higher level of arsenic in tube well water samples indicates that source of arsenic in water is natural rather than human activities. Dairy animals are mainly maintained in organized dairy farms and fed with chopped green fodder in Punjab. Arsenic concentration in fodder was reported to be higher in the arsenic contaminated area but the level was below the maximum permissible limit for fodders (Sidhu *et al.* 2012). Fodder seldom accumulates arsenic at concentrations fatal to animal because phytotoxicity results before such threshold concentrations are reached in plants (Virk 2020). Higher level of arsenic in fodder samples can be attributed to the increased use of arsenal pesticides in the agricultural fields and irrigation with the arsenic contaminated water. Increased fodder arsenic level could be a threat to the buffaloes because they are mainly fed with the green fodder. Higher arsenic level observed in the buffalo blood samples collected from the arsenic contaminated area could be due to the intake of arsenic mainly through contaminated drinking water and fodder which was supported by the close relationship observed between arsenic concentrations in tube well water, fodder and buffalo blood samples in the present study.

**Circulatory immune complexes and total immunoglobulins**

Level of circulatory immune complexes in buffaloes of the arsenic contaminated area was significantly ($p<0.05$) elevated with a decline ($p<0.05$) in the level of plasma total immunoglobulins compared to control area (Figure 1). Plasma CICs concentration was 2.96 fold increased whereas total

**Table 1.** Arsenic concentration (mean ± S.E.) in tube well water (µg/ml), fodder (µg/g) and buffalo blood (µg/ml) samples of control and arsenic contaminated area.

| Samples | Arsenic Concentration | |
|---|---|---|
| | Control Area (n=10) | Arsenic Contaminated Area (n=20) |
| Tube well water | 0.003±0.002 | 0.04±0.002* |
| Fodder | 0.02±0.003 | 0.29±0.01* |
| Buffalo blood | 0.02±0.001 | 0.12±0.006* |

*Indicates significant difference at p<0.05. Maximum permissible limit of arsenic in drinking water in view of animal health is 0.01µg/ml (WHO 2005). Phyto-toxicity limit of arsenic is 1 µg/g dry weight basis (Sidhu *et al.* 2012).

**Table 2.** Relationship between arsenic concentrations in tube well water, fodder and buffalo blood samples of arsenic exposed area (before *Terminalia arjuna* bark powder treatment)

| Parameters | Correlation coefficient (r) |
|---|---|
| Arsenic $_{(Tube\ well\ water)}$ Vs. Arsenic $_{(Fodder)}$ | 0.53* |
| Arsenic $_{(Tube\ well\ water)}$ Vs. Arsenic $_{(Buffalo\ blood)}$ | 0.791* |
| Arsenic $_{(Fodder)}$ Vs. Arsenic $_{(Buffalo\ blood)}$ | 0.626* |

*Indicates significant difference at p < 0.05; Number of each type of sample=20

**Table 3.** Relationship between blood arsenic level and immunological indices in buffaloes of arsenic contaminated area (before *Terminalia arjuna* bark powder treatment).

| Parameters | Pearson's correlation coefficient (r) |
|---|---|
| Arsenic $_{(Buffalo\ blood)}$ Vs. Plasma CICs | 0.212* |
| Arsenic $_{(Buffalo\ blood)}$Vs. Total plasma Igs | - 0.468* |

*Indicates significant relationships at p < 0.05; Number of each type of sample=20

immunoglobulins level was observed to be decreased by 2.22 fold in buffaloes of the arsenic contaminated area in comparison to control area. Blood arsenic level in untreated buffaloes was significantly correlated with circulatory immune complexes (r = 0.212, p<0.05) and plasma total immunoglobulins level (r = -0.468, p<0.05) in arsenic contaminated area (Table 3).

Chemical toxins can form immune complexes and have suppressive effect on the immune system. CICs have profound effect on humoral and cellular immune response through the receptors of immuno-competent cells. Permanent presence of CICs indicates abnormal immune regulation of an organism. Phagocytic cells such as neutrophils and macrophages remove the CICs from the organism. Persistent presence of CICs exhausts the neutrophils losingits function for a short period. Activation of glycolysis in neutrophils in an alkaline medium observed in exhausted neutrophils restores their function again Erythrocyte complement receptor (E-CR1)-C3b complex can bind to CICs which safely deliver these complexes to monocyte-phagocytic and reticuloendothelial system. This process prevents deposition of CICs in tissues (Mood *et al.* 2021). The increased concentration of immune complexes observed in buffaloes of arsenic contaminated area in present study could be a result of constant intake of arsenic through drinking water and fodder. Arsenic can form complexes with antibodies and deposited in the tissues.

The tissue injury caused due to free radical damage by arsenic releases the CICs to plasma as observed in the present study. CICs formation has also been reported in cattle exposed to environmental pollutant (Ramanaviciene *et al.* 2004) which is in accordance with the present study. The formation of CICs might be the reason for systematic antigen stimulation and reduction in activity of immune system. However, the mechanism of formation of CICs due to arsenic exposure is not clearly understood (Mood *et al.* 2021). Immunoglobulins are produced by plasma cells and act as a critical part of immune response by binding with specific antigens. The decreased total immunoglobulins observed in buffaloes of arsenic contaminated area in present study might be a consequence of disturbances in immune regulation of the organism and abnormal function of white blood cells due to formation of CICs. Significant correlation of blood arsenic level with total immunoglobulins and CICs in untreated buffaloes of arsenic contaminated area provided indication about the probable influence of this toxic metalloid on humoral and cellular immune response.

**Effect of *Terminalia arjuna* bark powder on the concentration of CICs and total immunoglobulins**

Treatment of arsenic exposed buffaloes with *Terminalia arjuna* bark powder @ 42mg/kg b. w. for 30 days resulted in significant (p<0.05) decrease in circulatory immune complexes in arsenic exposed buffaloes compared to control (Figure 2A). However, concentration of plasma total immunoglobulins was significantly (p<0.05) enhanced to the level compared to that of control after 15 days of treatment with *Terminalia arjuna* bark powder @ 42mg/kg b. w. (Figure 2B). This study observed that, treatment with *Terminalia arjuna* bark powder cured the arsenic mediated adverse effects on immune status of buffaloes. The curative effects were also maintained in buffaloes upto day 45 as evidenced from the increased total immunoglobulins and decreased circulatory immune complexes level in 45$^{th}$ day plasma sample of *Terminalia arjuna* bark powder treated buffaloes compared to the corresponding controls.

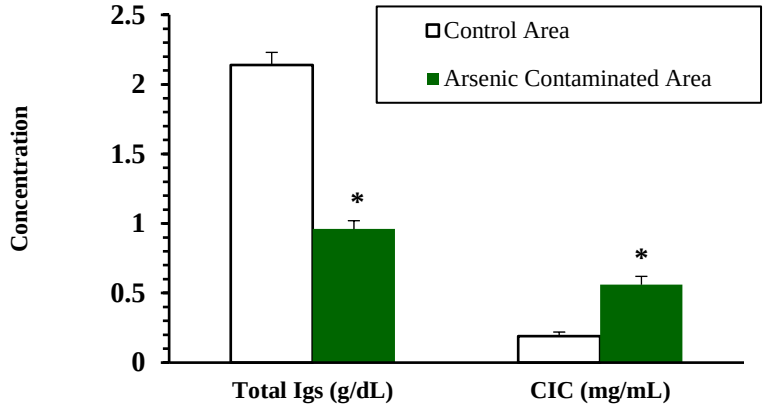
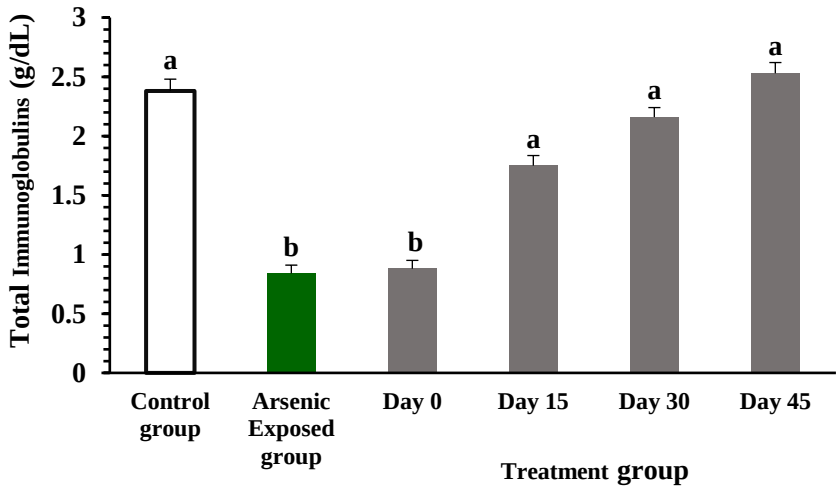
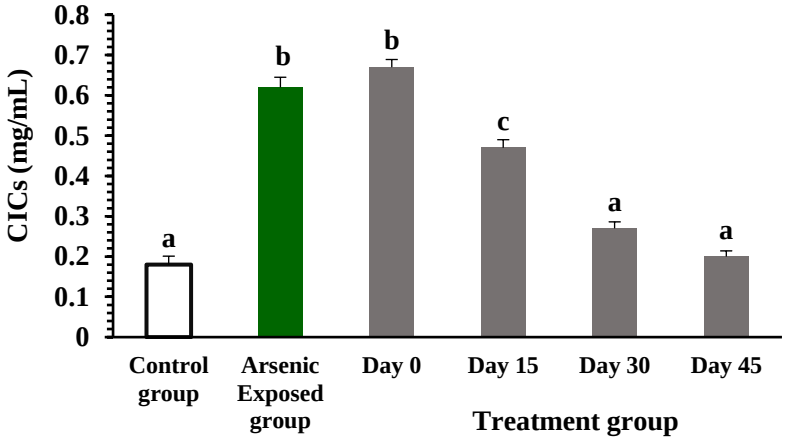



**Fig. 1:** Concentration (mean ± S.E.) of plasma total immunoglobulins and circulatory immune complexes (CIC) in buffaloes of control and arsenic contaminated area. *Indicates significant ($p<0.05$) difference from corresponding controls.




(A)




(B)

**Fig. 2:** Concentration (mean ± S.E.) of (A) plasma total immunoglobulins and (B) circulatory immune complexes in arsenic exposed buffaloes supplemented with *Terminalia arjuna* bark powder. Columns superscripted with different letters are significantly different at $p<0.05$.

The curative effect of *Terminalia arjuna* bark powder on the level of CICs and total immunoglobulins could be due to its immunomodulatory activity. *Terminalia arjuna* bark extract has been reported to improve the neutrophil function in humans (Ramesh and Palaniappan 2023 and Jalalpure *et al.,* 2006). Previous research on mice and rats suggested that *Terminalia arjuna* bark powder has immunomodulatory potential (Halder *et al.* 2009). Saponins present in *Terminalia arjuna* bark have been reported to possess immuomodulatory activity and stimulate the lymphocytes *in vitro* (Ramesh and Palaniappan 2023). The exact mechanism by which the compounds present in *Terminalia arjuna* bark eliminate the CICs is still not clear. The decrease in concentration of CICs followed by *Terminalia arjuna* bark powder treatment might be due to activation of phagocytic activity, macrophages or erythrocyte complement receptor (Mood *et al.* 2021) which is in line with the results of this study. Improved plasma cell function and efficient immune regulation by the *Terminalia arjuna* bark powder treated buffaloes might be the reason for the improvement in plasma total immunoglobulins status (Halder *et al.* 2009) which was observed in this study.

**Conclusion**

Exposure to arsenic contaminated water and fodder altered the levels of circulatory immune complexes and plasma total immunoglobulins in murrah buffaloes. Treatment with *Terminalia arjuna* bark powder @ 42mg/ kg. b.w. cured the arsenic mediated disturbances in circulatory immune complexes and plasma total immunoglobulins concentration in buffaloes compared to that of control. The curative effects were maintained in *Terminalia arjuna* bark powder treated buffaloes as evidenced from the improved immunity status on day 45. The bark of this plant could be very useful for improving the immune status of buffaloes. However, more studies are required to identify and characterize the compounds present in *Terminalia arjuna* bark that is responsible for these curative effects.

**Conflict of interest** No conflict of interest.

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