



Cadmium induced oxidative stress-mediated pathophysiological alterations in chickens and their amelioration by polyherbal mixture enriched feed

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The study was conducted to evaluate sub-acute toxicity of cadmium (Cd) in broiler chickens and its amelioration by polyherbal mixture enriched feed (PHMEF). Thirty broiler chickens were divided into five groups. Chickens of group 1 were kept as control (C1). Chickens of group T1, T2, T3 and T4 were exposed to cadmium (as cadmium chloride) through drinking water (100 ppm), Vitamin C with Vitamin E (250 mg/kg each) in feed + Cd in drinking water (100 ppm), PHMEF (2%) alone, and PHMEF (2%) + cadmium in drinking water (100 ppm) for 28 days, respectively. Haematology, serum biochemical parameters, oxidative stress parameters like superoxide dismutase, catalase, reduced-glutathione and malondialdehyde, cadmium accumulation in organs like kidney, liver and thigh muscle, scanning electron microscopy (SEM) of ileum, and histopathological examination of kidney, liver and intestine were performed to evaluate toxicity of cadmium and ameliorating potential of PHMEF in broiler chickens. PHMEF modifies biochemical parameters, oxidative stress markers. There was a significant reduction of cadmium accumulations in the PHMEF treated group. Polyherbal mixture enriched feed showed ameliorating effect against cadmium induced toxicity due to antioxidant effect as well as effect on accumulation of the cadmium in chickens.

Keywords: Broiler chickens, Cadmium toxicity, LC-QToF-MS analysis, Polyherbal mixture enriched feed, Scanning electron microscopy

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Development of the mining sector and indiscriminate industrialization has increased the level of cadmium (Cd) in water and soil. Cadmium enters in plants from soil and accumulates in different parts¹. Aquatic animals accumulate Cd which can be considered as an important source of Cd intoxication in poultry which usually fed with fish meal for calcium supplementation². The poultry industry in India is facing the problem of reduction in growth and production in stress condition. Stress due to heat, cold, heavy metals and other environmental contaminants may affect overall performance in poultry. The residues of Cd at a low level in the feed of chickens adversely affect cytochrome P450 dependent monooxygenase system³. It is also reported that Cd in drinking water has induced oxidative stress in poultry⁴. Ascorbic acid supplementation protect the broiler birds from Cd-induced lipid peroxidation⁵. Alternatively, herbal plants are a rich source of active constituents having antioxidant potential and can be

used to protect oxidative damage. Antioxidant effect of *Opuntia elatior* Mill.⁶, *Peltophorum pterocarpum* (DC) Baker ex DC⁷, *Syzygium cumini* (L.) Skeels⁸, *Withania somnifera* (L.) Dunal⁹, *Sphaeranthus indicus* (L.)¹⁰, *Cressa cretica* (L.)¹¹ and *Solanum xanthocarpum* Schrad. & Wendl¹² have been previously evaluated. Based on previous reports and *in vitro* evaluation of antioxidant property of plants, the present study was planned to prepare polyherbal mixture enriched feed (PHMEF) from different plant material and to evaluate its ameliorating potential against Cd-induced oxidative stress in chickens.

Materials and Methods

Preparation of polyherbal mixture

All the plant materials were collected from a local area of Junagadh, Campus of Gujarat Ayurveda University and coastline of Jamnagar district (Table 1). All the plant specimens were authenticated by Dr R. C. Viradia (Botanist), Professor, Department of Biology, Bahauddin Science College, Junagadh, India. Each plant material was subjected to shade dry

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Table 1 — List of various plant material used to prepare polyherbal mixture

Sr. No.	Botanical name	Vernacular name	Part of plant
1	<i>Opuntia elatior</i> Mill.	Hathlo thor	Fruit
2	<i>Peltophorum pterocarpum</i> (DC) Baker ex DC	Pilo gulmohor	Leaves
3	<i>Syzygium cumini</i> (L.) Skeels	Kala-jambu	Leaves
4	<i>Withania somnifera</i> (L.) Dunal	Ashwagandha	Leaves
5	<i>Sphaeranthus indicus</i> (L.)	Gorakh mundi	Fruit
6	<i>Cressa cretica</i> (L.)	Rudanti	Leaves
7	<i>Solanum xanthocarpum</i> Schrad. & Wendl.	Bhoi-ringani	Aerial part

and used to make fine powder which was stored in airtight containers for further use. Equal amount of powder of each plant material was mixed to make the polyherbal mixture (PHM).

Preparation of polyherbal mixture enriched feed and vitamin-enriched feed

The PHMEF was prepared by mixing 2 g polyherbal mixture per 100 g (2% w/w) of standard poultry starter and growing feed. Vitamin C and E (HiMedia Pvt. Ltd., Mumbai) were used as standard antioxidant compounds¹³ which were mixed with feed to prepare a vitamin-enriched feed. Vitamin E was dissolved in sunflower oil and vitamin C was dissolved in distilled water at the concentration of 250 mg per 4 mL. The vitamin-enriched poultry feed was prepared by mixing 4 mL of vitamin E and vitamin C solutions in 1 kg feed in a mixer grinder. The final strength of Vitamin C and E was 250 mg/kg of poultry feed each. The nutritional analysis of both the feed was also carried out (Supplementary Table S1)

Procurement of chicks, husbandry practices and ethical approval

Thirty day old broiler chickens were procured from Venky's India Pvt. Ltd., Anand, Gujarat, India. All broiler chickens were randomly selected for the study at the age of 3 weeks. The broiler chickens were housed in a well-ventilated room (Temperature: 24 to 28°C, Relative humidity: 40 to 55%) with the provision of an air-conditioner. Vaccination was followed as per standard procedure to control common infectious diseases of chickens. Poultry starter feed was provided *ad libitum* to chickens up to four weeks of age and then broiler finisher feed was given. Reverse osmosis (RO) drinking water was provided freely. The necessary steps including sanitization and changing of bedding material were taken to keep the chickens stress free. The animal experiment was approved by IAEC, College of Veterinary Science & AH, JAU, Junagadh vide Protocol No. JAU/JVC/IAEC/SA/25/2017.

Grouping of chickens and types of treatments

At the age of two weeks, the chickens were grouped as per their body weight (six chickens in each) in following groups; Control 1 (C1), Treatment 1 (T1), Treatment 2 (T2), Treatment 3 (T3), Treatment 4 (T4). Chickens of group 1 were kept as control (C1) provided normal feed and drinking water. Chickens of group T1 were exposed to cadmium (as cadmium chloride) through drinking water (100 ppm), Chickens of group T2 were exposed to Vitamin C with Vitamin E (250 mg/kg each) in feed + cadmium in drinking water (100 ppm), Chickens of group T3 were exposed to PHMEF (2%) alone and Chickens of group T4 were exposed to PHMEF (2%) + cadmium in drinking water (100 ppm) for 28 days. Weekly body weight of chicken was recorded. The feed consumption was also recorded daily. Different types of treatments were assigned to each group for 28 days.

Collection of samples and analyses

At the end of the experiment, from wing vein of each bird, 1.5 mL blood samples were collected in a sterile anticoagulant containing vials and centrifuged to separate plasma while serum was separated by collecting 1 mL blood in anticoagulant free vials for antioxidant and biochemical analysis. The chickens were sacrificed humanly and visceral organs like liver, kidney and intestine were collected for gross and histopathological examination. A small part of the tissue of the liver, kidney as well thigh muscle was collected separately in glass petri-plate for the estimation of Cd accumulation. For Scanning Electron Microscopy (SEM), ileum part of the intestine was taken in 0.1 M phosphate buffer (pH 7.4). About 0.5 to 1 g tissue of the liver and kidney was collected in a buffer solution for the estimation of oxidative stress parameters.

Haematology and biochemical parameters

Haemoglobin (Hb) estimation was carried out by Sahli's hemoglobinometer and expressed as gram per

cent, PCV was estimated by the microhematocrit method and expressed as per cent¹⁴. Estimation of TLC and TEC was carried out as per standard method¹⁵. Semi-automatic biochemistry analyser (Diatek Healthcare Pvt. Ltd., India) was used for estimation of biochemical parameters using standard kits (Diatek Health care Pvt. Ltd., India).

Evaluation of parameters of oxidative stress

The serum, plasma, RBC lysate, liver and kidney were used for the estimation of various oxidative stress parameters like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and MDA level. Other chemicals of analytical grade were purchased from Sigma Aldrich, USA, Merck Ltd., Mumbai and S.D. fine Chemicals, Mumbai.

Preparation of serum sample

Fresh blood was collected from wing vein in anticoagulant free vials and allowed to clot the blood for 5 min and centrifuged at 10,000 rpm at 4°C for 5 min. Separated serum was collected in another tube and used for determination of SOD activity.

Preparation of RBC lysate

Catalase activity was estimated from blood lysate which was prepared by mixing 450 µL of RBC lysis buffer with 50 µL of blood sample for 5 min.

Preparation of plasma sample

Whole venous blood collected in the anticoagulant vial was centrifuged at 10,000 rpm for 10 min and plasma collected from each sample was used for lipid peroxidation assay.

Preparation of tissue sample

Tissue samples of liver and kidney (100 mg) from each chicken was collected and immediately stored in ice-cold 0.1 M (1 mL) phosphate buffer saline (pH: 7.4) for estimation of CAT and GSH, whereas 0.5 g sample of both organs was separately collected in 1 mL of 10 mM Tris, 1mM EDTA buffer (pH: 8.2) for analysis of SOD. Tissues were homogenized followed by centrifugation at 10,000 rpm (4°C) for 10 min and the supernatant from each sample was used for determination of catalase activity.

Determination of SOD activity

Superoxide dismutase activity in serum and tissue samples was determined as per standard method¹⁶. For serum samples, 2.8 mL of Tris-EDTA buffer (pH-8.2) was mixed with 0.1 mL of the serum sample, mixed well. After adding 0.1 mL of pyrogallol solution (20 mM) the reaction was started and reading was taken at 420 nm after 1.5 and 3.5 min. An absorbance

difference per 2 min was recorded. For control, the sample was replaced by buffer solution and readings were taken against buffer as a blank. The SOD activity was calculated using following formula;

$$\begin{aligned} \text{Units of SOD per 3 mL assay mixture} \\ = \frac{(A - B) \times 100}{(A \times 50)} \end{aligned}$$

Where, A=absorbance of control; B=absorbance of sample

$$\text{Unit} \times 10 = \frac{\text{units}}{\text{mL of a sample solution}}$$

One unit of superoxide dismutase is the enzyme leads to 50% pyrogallol auto-oxidation inhibition per 3 mL of mixture.

Tris-EDTA buffer (pH-8.2) was used to prepare tissue homogenates which were centrifuged for 40 min at 10000 rpm (4°C). The supernatant was used for the assay. Tris-EDTA (2900 µL) was mixed with pyrogallol (2 mM, 100 µL) in the cuvette and scanned for 3 min at 420 nm wavelength. Then 2890 µL Tris-EDTA buffer (pH-8.2), 100 µL pyrogallol and 10 µL of tissue homogenate were taken and scanned for 3 min at the same wavelength. The SOD activity was expressed as Units/mg protein/minute.

The enzyme unit can be calculated using the following equations:

$$\% \text{ inhibition} = \frac{(A - B) \times 100}{B}$$

Where, A=absorbance of control; B=absorbance of sample

$$\text{Whereas, Enzyme unit (U)} = \left(\frac{\% \text{inhibition}}{50} \right) \times \text{dilution factor (100)}$$

Determination of catalase activity

Tissue and blood catalase activity was determined using standard method¹⁷. Twenty µL of blood lysate was mixed with 1980 µL PBS (0.1 M PBS, pH 7.5) in a test tube. Then 1 mL of 30 mM H₂O₂ was added to it and the absorbance of the reaction was taken at 240 nm for 1 min, against blank having a mixture of PBS and blood lysate only without H₂O₂. The unit activity of CAT was expressed in a molar/minute. About 20 µL of tissue homogenate was mixed with 1980 µL PBS (0.1 M, pH 7.4). Then 1 mL of H₂O₂ (30 mM) was mixed to it and the absorbance of the test sample was measure at 240 nm against blank with a

mixture of PBS and homogenate. The molar extinction coefficient of 43.6 cm^{-1} was used to calculate activity of catalase. Protein estimation in organ tissue samples was carried out using the standard method¹⁸ and used to calculate catalase activity.

$$\begin{aligned} & \text{mmoles of Hydrogen peroxide decomposed} \\ & \quad / \text{minute/ mg protein} \\ & = \frac{(\Delta A/\text{min}) \times 1000 \times 3}{43.6 \times \text{mg protein in a sample}} \end{aligned}$$

Determination of reduced glutathione level

Reduced glutathione (GSH) was measured from liver and kidney tissue samples using the standard kit (Sigma Aldrich, Germany). Briefly, 100 mg tissue samples (liver and kidney) were collected and deproteinized using 1 mL of 5% sulfosalicylic acid. The masking reagent was used to determine the oxidized form of glutathione (GSSG). The final concentration of reduced glutathione was expressed in μM .

Estimation of malondialdehyde level

Lipid peroxidation was measured as a malondialdehyde level using the standard kit (Sigma Aldrich). In procedure, 10 μL of plasma samples from all groups were deproteinized using 500 μL of 42 mM sulfuric acid, 125 μL of 10% phosphotungstic acid. The content was centrifuged at 14,000 rpm for 3 min. The pellet thus formed was suspended in Butylated Hydroxy Toluene (BHT) solution to get the sample. Six hundred microliter thiobarbituric acid (TBA) solution was added to form an adduct of TBA-MDA. To enhance the sensitivity of the estimation, 300 μL of n-butanol was added to the adduct solution followed by addition of 100 μL of 5M NaCl solution. Centrifuge the tubes at 14,000 rpm for 3 min. The supernatant (500 μL) was taken in 96-well plate and read at 534 nm in a spectrophotometer. The MDA level in plasma of various treatment groups was measured using a calibration curve of standard MDA (0-100 μM). The final concentration of MDA was expressed in nanomole.

Detection of cadmium level in liver, kidney and muscle

The Cd accumulation in liver, kidney and muscle tissue of chickens was detected by dry ash method¹⁹ using MP-AES (Microwave Plasma Atomic Emission Spectroscopy, Agilent-4200 model). Collected tissues of liver, kidney and muscle were dried at 105°C to make fine powder. About 1 g powder of each organ was taken into silica crucible and heated to 550°C for 5 h in a muffle furnace. After cooling down of muffle furnace the ash scraped and to these, 5 mL tri-acid mixture ($\text{HNO}_3:\text{HClO}_4:\text{H}_2\text{SO}_4 - 6.5:6:2$) added. The

heat treatment was given to samples until a clear solution obtained. The solution was diluted to 50 mL with distilled water and designated as stock-1. From the stock-1 solution, 5 mL solution was taken in a separated 15 mL polypropylene tube and pH 6.0 was adjusted with 25% ammonia solution (stock-2). The final volume of 10 mL was made with distilled water. The stock-2 was used for the detection of Cd. The Cd level was expressed in μg per gram of tissue.

Scanning Electron Microscopy (SEM) of the ileum

A piece of ileum part of the intestine was taken in 1.5 mL of 0.1M phosphate buffer immediately after the sacrifice of each chicken. It is made free from food debris by washing three times with 0.1M phosphate buffer solution. After washing tissues were cut in rectangular shape and fixed in 2.5% glutaraldehyde (prepared in 0.1 M phosphate buffer) for 24 h. After fixation, tissue was again washed with 0.1 M phosphate buffer to remove the fixative and stored in 0.1 M phosphate buffer until use. For SEM, tissue was dehydrated using varying concentrations of acetone in increasing order like 30% to 100% acetone²⁰. Dehydrated tissue was stored in air-tight moisture free vial with calcium chloride. Scanning Electron Microscopy was performed using a Zeiss EVO-18 instrument by coating samples with strips of carbon and gold particle on stainless steel stab. To stabilize the samples and increase the resolution, samples were impregnated with the agar solution. The prepared samples have been mounted in the vacuum chamber of SEM. The electron beam was set to 15 kV to get the high-resolution photographs.

Histopathological evaluation of organs

Buffered formalin (10%) was used to collect the liver, kidney and intestine tissue for histopathological examination. After paraffin wax embedding and tissue sectioning (5 μ), section of each tissue were stained with haematoxylin and eosin (H & E) stain²¹. The slides were observed for microscopic pathological lesions.

LC-QToF-MS analysis of hydroalcoholic extract of polyherbal mixture

Accurately weighed 10 g of the polyherbal mixture (PHM) was extracted with 100 mL of 50% methanol on ultrasonic bath for 30 min at room temperature and allowed to stand for a day. The extract was filtered off and dried in a rotary evaporator. The total yield of hydro-alcoholic extract was 14.11 % w/w. Separation of sample was carried out with the chromatographic system (Agilent Technologies, USA, Model 6540)

with C₁₈ column (4.6 × 100 mm, 3.5 μm) at 25 °C. The method used a gradient at constant flow rate (0.6 mL min⁻¹) combining solvent A (0.1% formic acid/water) and solvent B (acetonitrile), programmed as follows: 0 min, linear change from A–B (95:5 v/v) to A–B (5:95 v/v); 12 min, isocratic A–B (5:95 v/v); 20 min, linear change to A–B (95:5 v/v) 22 min and 25 min, linear change to A–B (95:5 v/v). The 10 μL extract was injected in the sample injector.

MS/MS analysis was done with Ultra-High-Definition Accurate-Mass QToF-MS with LC, (Drying gas flow (Nitrogen): 10.0 L min⁻¹, nebulizer pressure: 45 psi, gas drying temperature: 325°C, Gas vaporize temperature: 350°C capillary voltage: 0.051 μA, chamber voltage: 4.23 μA). Fixed collision energies were set to 10, 20, 30, 40, 50 V, precursors per cycle were set to 5 and precursor threshold was 400 counts, mass scan range: 100-1700 m/z and scan speed was 25000 counts/spectrum. Data were analysed using Mass Hunter software (Agilent Technologies, USA) along with METLIN Personal Compound Database with accurate mass MS/MS Library (PCDL).

Statistical analysis

All numerical data were expressed in mean ± Standard Error of Mean (SEM). Data were analyzed by one-way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) to compare the difference in means with *p*-value < 0.05 considered statistically significant²².

Results and Discussion

Weekly average body weight (g) of chickens under different treatment groups are depicted in Supplementary Table S2. At the end of the experiment, values of body weight of chickens under control group (C1) and chickens treated with only PHMEF (T3) were significantly (*p*<0.05) higher as compared to other treatment groups. Chickens treated with vitamin-enriched feed along with Cd in the group (T2) showed significant (*p*<0.05) higher body weight as compared to chickens exposed to Cd only (T1). However, the body weight of chickens treated

with PHMEF along with Cd (T4) was non-significantly higher than that of chickens exposed to Cd only (T1). The data indicate that polyherbal mixture in feed reduced the toxic effect of Cd on body weight gain. Cd administration has also been reported to cause decrease in growth rate and resulted in to decrease in body weight in the chickens²³. Cd depresses feed intake in broiler chickens might be due to oxidative stress²⁴. The feed consumption data (g/bird/day) are shown in Supplementary Table S3. At the end of the experiment, group of chickens treated with PHMEF along with Cd (T4) showed significantly (*p*<0.05) high feed consumption among all treatment groups followed by vitamin-enriched feed with Cd in the group T2 and control group (C1). However, chickens treated with only Cd (T1) showed significantly (*p*<0.05) low feed consumption among all treatment groups which indicates the direct effect of Cd on the gastrointestinal tract.

Haematological parameters of different treatment groups are depicted in Table 2. At the end of the experiment, haemoglobin (g/dL) in a group of chickens treated Cd (T1) was significantly lower and that in chickens treated with vitamin-enriched feed with Cd (T2) was significantly (*p*<0.05) higher among all treatment groups. Chickens treated with only PHMEF (T3), PHMEF along with Cd (T4) and control group (C1) showed significantly (*p*<0.05) higher Hb level as compared to chickens exposed to Cd only (T1). The results indicate that the polyherbal mixture in feed might reverse the effect of Cd on haemoglobin synthesis. However, it was previously reported that the Hb level in chickens was unaltered after exposure to a low level (50 ppm) of Cd²⁵. Pack cell volume (%) in a group of chickens exposed to Cd only (T1) was significantly higher amongst all treatment groups followed by vitamin C and E along with Cd (T2). PCV% in chickens of the control group (C1), chickens treated with PHMEF only (T3) and chickens treated with PHMEF along with Cd (T4) were significantly (*p*<0.05) lower as compare to toxicity group (T1). Total erythrocyte count (×10⁶/cmm) count was not significantly (*p*>0.05)

Table 2 — Haematological parameters in chickens of different treatment groups

Parameters	C1	T1	T2	T3	T4
Hb (g/dL)	8.60±0.19 ^b	7.07±0.12 ^a	9.10±0.04 ^c	8.97±0.06 ^c	8.93±0.07 ^c
PCV (%)	29.33±1.69 ^a	34.33±2.43 ^b	31.50±1.06 ^{ab}	28.83±0.79 ^a	28.83±1.17 ^a
TEC (×10 ⁶ /cmm)	1.74±0.08 ^b	1.69±0.18 ^a	2.11±0.14 ^b	1.98±0.04 ^{ab}	1.99±0.11 ^{ab}
TLC (×10 ³ /cmm)	17.38±1.75 ^a	17.62±2.61 ^a	15.24±1.24 ^a	16.15±1.42 ^a	14.77±1.84 ^a

Values with different superscripts in each row are significantly different (*p*<0.05.)

affected in chickens following exposure to Cd. This finding supports less effect of Cd on RBC's in chickens at the level of exposure used in the study which might be due to the body's defence mechanism against the low level toxicity.

Serum biochemical parameters of different treatment groups at the end of the experiment are depicted in Table 3. Serum cholesterol level (mg/dL) was significantly ($p<0.05$) decreased in chickens treated with only Cd (T1) as compared to control group (C1). Whereas, chickens treated with PHMEF along with Cd (T4) showed significantly ($p<0.05$) high serum cholesterol level as compared those of all treatment groups. A serum triglyceride level in the Cd-exposed group (T1) was slightly lower as compared to control (C1). The level of triglyceride in T2, T3 and T4 groups were non-significantly higher and comparable to that of control chickens. Cd-exposed group (T1) showed a significantly low level of serum HDL and LDL as compared to all the treatment groups. Serum HDL and LDL levels (mg/dL) in the group treated with PHMEF along with Cd (T4) were almost normal. Decrease levels of cholesterol and triglyceride in Cd-exposed chickens indicate lipid peroxidation which was prevented by PHMEF treatment when given along with Cd exposure.

Serum ALT and AST (U/L) level in Cd-exposed group (T1) was slightly higher (non-significantly) as compared to those of all treatment groups. ALP (U/L) in chickens treated with PHMEF along with Cd (T4) and the group treated with Cd only (T1) were not significantly altered in treatment groups. The level of total protein, albumin, urea and uric acid in chickens

of different treatment groups were within a normal range. Serum LDH level (U/L) in the Cd-exposed group (T1) was higher (Non-significantly) amongst all groups. However, LDH levels in chickens treated with PHMEF along with Cd (T4) and vitamin C and E along with Cd (T2) were comparable to that of normal control chickens.

Various oxidative stress parameters from blood samples of different treatment groups at the end of the experiment are presented in Table 4. Polyunsaturated fatty acids in cells are converted into MDA upon peroxidation. MDA overproduction due to free radicals is an indicator of cell oxidative injury^{26,27}. Cd enhances adverse effects in various organs via oxidative stress induction. With Cd dietary overload, peroxidation of membrane lipids causes liver and kidney injury by free radical generation, as evidenced by an increase in MDA production²⁸. Plasma MDA level in the chickens exposed to Cd only (T1) was significantly ($p<0.05$) higher compared to normal control (C1). The plasma MDA level in the group treated with vitamin C and E enriched feed along with

Table 4 — Various antioxidant parameters from plasma, serum and RBC lysate

Groups	Plasma MDA (nM)	Serum SOD (U/mL)	RBC lysate Catalase (molar/minute)
C1	87.33±32.83 ^a	9.58±1.67 ^{ab}	0.61±0.14 ^a
T1	217.33±97.25 ^b	12.50±1.12 ^b	0.54±0.10 ^a
T2	84.00±33.07 ^a	9.79±1.27 ^{ab}	0.80±0.14 ^a
T3	77.33±22.90 ^a	10.21±0.99 ^{ab}	0.72±0.10 ^a
T4	140.67±39.47 ^a	7.29±1.46 ^a	0.74±0.06 ^a

Values with different superscripts in each row are significantly different ($p<0.05$.)

Table 3 — Biochemical parameters in chickens of different treatment groups

Parameters	C1	T1	T2	T3	T4
Glucose (mg/dL)	247.00±6.76 ^a	245.50±5.25 ^a	274.83±5.25 ^b	256.83±2.96 ^a	258.50±4.22 ^a
Cholesterol (mg/dL)	160.33±6.27 ^{bc}	124.17±8.65 ^a	148.17±5.92 ^b	156.17±5.75 ^{bc}	172.50±4.06 ^c
Triglyceride(mg/dL)	50.17±5.90 ^a	38.67±5.46 ^a	47.33±2.93 ^a	55.17±10.66 ^a	49.00±3.85 ^a
HDL (mg/dL)	119.18±5.32 ^{bc}	95.97±6.44 ^a	111.06±4.38 ^b	115.44±3.99 ^b	133.75±2.91 ^c
LDL (mg/dL)	49.39±3.06 ^b	37.14±2.58 ^a	44.97±2.48 ^{ab}	47.61±2.69 ^b	48.77±2.79 ^b
ALT (U/L)	5.56±0.97 ^a	6.42±0.62 ^a	4.02±0.50 ^a	4.80±0.74 ^a	4.81±0.92 ^a
AST (U/L)	364.85±36.22 ^a	373.33±20.05 ^a	368.93±22.61 ^a	336.91±19.46 ^a	353.23±24.71 ^a
ALP (U/L)	42.65±13.91 ^a	39.33±9.38 ^a	55.23±14.60 ^a	54.35±15.84 ^a	42.51±9.60 ^a
LDH (U/L)	750.00±45.96 ^a	981.19±188.91 ^a	749.17±115.02 ^a	729.25±59.87 ^a	781.07±103.05 ^a
Protein (gm/dL)	2.94±0.11 ^a	3.09±0.14 ^a	2.92±0.08 ^a	2.96±0.09 ^a	3.03±0.07 ^a
Albumin (gm/dL)	1.44±0.03 ^{ab}	1.50±0.04 ^b	1.38±0.04 ^a	1.47±0.03 ^{ab}	1.46±0.03 ^{ab}
Uric acid (mg/dL)	4.11±0.37 ^a	3.89±0.46 ^a	3.37±0.26 ^a	4.14±0.15 ^a	3.93±0.33 ^a
Urea (mg/dL)	4.72±0.57 ^a	4.75±0.29 ^a	4.07±0.20 ^a	4.74±0.38 ^a	4.85±0.11 ^a

Values with different superscripts in each row are significantly different ($p<0.05$.)

Cd (T2) and the group treated with PHMEF only (T3) were found to be at par with the control group (C1). PHMEF along with Cd was able to reduce the plasma MDA level as compared to the Cd-exposed group. The result clearly indicates the protective effect of PHMEF against Cd-induced lipid peroxidation. Higher activity of serum SOD was observed in chickens exposed to Cd only (T1) and that was lowest in PHMEF treatment group (T4). Generally, the SOD activity in toxicity is decreased after exposure to a higher level of xenobiotics due to protein damage but in the present study, lower level exposure to Cd caused the continuous generation of superoxide radicals which resulted in higher activity of SOD. Catalase activity in RBC lysate in chickens of the group treated with Cd only (T1) was lowest as compared to all other treatment groups which indicated that there was less conversion of hydrogen peroxide into the water which might be responsible for oxidative damage in chickens exposed to Cd only (T1). Catalase activity in chickens treated with vitamin C and E enriched feed along with Cd in the group (T2), only PHMEF treated group (T3) and PHMEF along with Cd in the group (T4) were higher as compared to chickens exposed to Cd only (T1). The result indicates that PHMEF affected the activity of catalase at a cellular level which can transform generated H₂O₂ into H₂O which is responsible for further damage in the body.

The activity of oxidative enzymes like SOD, catalase and GSH level in liver and kidney tissue are depicted in Table 5 and Table 6, respectively. SOD activity (U/mL) in liver and kidney tissues of chickens exposed to Cd only as well as PHMEF along with Cd were increased as compared to those found in respective tissues from chickens of normal control indicated continuous stimulation by producing the

free radicals by Cd which stimulated the body's defence mechanism (production of SOD) to convert the generated superoxide radicals into hydrogen peroxide. The SOD activity in liver and kidney of Vitamin C and E enriched feed treated chickens were significantly lower as both vitamins possess the direct scavenging effect on generated free radicals. PHMEF also produced a significantly higher level of SOD along with significantly increased levels of GSH which could be able to convert superoxide to H₂O₂ and H₂O₂ to water which might be the reason for less damage as compared to toxicity group (T1).

Catalase, an antioxidant enzyme is produced in all living organisms and it protects the cell from reactive oxygen species. The effects of the Cd exposure on CAT activity have been studied extensively and it has been established that the activity of CAT depends on the experimental conditions²⁹. Catalase activity (U/mg protein) in liver and kidney tissues of chickens exposed to Cd only (T1) were non-significantly ($p>0.01$) and significantly ($p<0.05$) lowered, respectively as compared to those of the normal control group (C1). Catalase activity in both tissues in PHMEF along with Cd exposure group (T4) were slightly higher compared to the Cd-exposed group (T1). This changes indicated higher conversion of produced H₂O₂ in H₂O. However, catalase activity in liver and kidney of chickens exposed to Cd in the diet was also reported to be significantly lower compared to the control group²⁵. This difference in the level or activity of enzymes may be due to variation in exposure level and duration.

The level of GSH (μ M) in liver and kidney tissues in the Cd-exposed group (T1) did not significantly differ from that of the normal control group (C1) along with a significant increase in SOD and decreased in catalase which might be the responsible

Table 5 — Various antioxidant parameters of liver of chickens of different treatment groups

Parameter	C1	T1	T2	T3	T4
SOD (U/mL)	12.75±2.70 ^a	22.06±5.41 ^{ab}	17.16±4.05 ^{ab}	19.12±2.92 ^{ab}	27.94±3.29 ^b
Catalase (U/mg protein)	21.88±7.95 ^a	12.01±3.22 ^a	14.41±4.97 ^a	10.53±1.98 ^a	14.72±6.57 ^a
GSH (μ M)	3.97±0.75 ^a	3.31±1.05 ^a	8.40±1.96 ^b	16.51±3.20 ^b	8.42±3.28 ^b

Values with different superscripts in each row are significantly different ($p<0.05$.)

Table 6 — Various antioxidant parameters of kidney of chickens of different treatment groups

Parameter	C1	T1	T2	T3	T4
SOD (U/mL)	18.63±5.13 ^a	24.51±3.77 ^{ab}	18.14±3.51 ^a	24.02±3.98 ^{ab}	34.80±4.52 ^b
Catalase (U/mg protein)	25.59±5.28 ^b	14.37±1.53 ^a	9.68±1.31 ^a	16.88±2.75 ^{ab}	17.41±1.50 ^{ab}
GSH (μ M)	1.89±0.13 ^a	2.69±0.44 ^a	8.49±1.82 ^b	12.24±1.77 ^c	9.72±1.28 ^b

Values with different superscripts in each row are significantly different ($p<0.05$.)

for more damage by Cd. However, the level of GSH (μM) in both tissues from the group treated with vitamin-enriched feed (T2), PHMEF (T3) and PHMEF along with Cd (T4) were significantly ($p < 0.05$) higher as compared to that observed in toxicity group (T1). The lowered GSH levels in Cd-exposed chickens was might be due to the binding of Cd to GSH for its excretion through bile as complex. Bharvi *et al.* (2011) also observed significantly decreased GSH level in kidney and liver of chickens exposed to 100 ppm Cd for 28 days which were significantly increased when broiler chickens were fed with 0.1% adaptogens in the feed³⁰. Gamma-glutamylcysteine synthetase and glutathione synthetase have role in synthesis of glutathione³¹. It has also been proved that flavonoids stimulate transcription of a critical gene for GSH synthesis in cells³². In the present study, significant increased level of GSH indicated that there was modulation of GSH concentration by PHMEF as it contains flavonoids. Flavonoids modulate GSH-dependent cellular processes³³.

Cadmium levels in various tissues (kidney, liver and muscle) were also determined and are presented in Table 7. The level of Cd deposition was highest in the kidney followed by liver and muscle. Vitamin treatment did not affect the level of Cd ($\mu\text{g}/\text{gm}$ tissue) in kidney and muscle of chickens. However, the level of Cd in liver tissue of the vitamin-enriched feed treated group was non-significantly lowered as compared to that of Cd-exposed group (T1). The highest Cd level was also reported elsewhere in kidney, followed by the liver and the lowest Cd levels were found in leg muscle of broiler chickens³⁴. Surprisingly, the levels of Cd in kidney and muscle tissues of chickens treated with PHMEF with Cd (T4) in the present experiment were significantly ($p < 0.05$) reduced as compared to Cd-exposed control group (T1) which indicates a probable role of adaptogens in the excretion of the Cd from the body. Results of the present study indicate that dilatory supplementation of the PHMEF reduced the Cd accumulation in kidney and muscle and protected them from subsequent Cd-induced oxidative alterations by free radicals. Herbal

antioxidants with oxygen free radicals scavenging effect which averted the GSH exhaustion. There might be possibility of chelation of Cd by herbal antioxidants³⁵ and increased its elimination.

Scanning Electron Microscopy (SEM) of intestine from chickens of various treatment groups showed the clear and three-dimensional view of the changes in the villi and microvilli of the intestine (ileum) (Fig. 1A to Fig. 1J). In the normal control group (C1), tongue-shaped villi with finger-like projections were observed without breakage. The surface of villi showed transverse folds along with the corrugated surface. The corrugated surface of villi on higher magnification exhibited almost continuous uniformity and no disruption of the epithelial mucosa (Fig. 1A). The tip of villi has been observed for cell protuberance. Examination of villi on higher magnification, the microvilli appeared as knob-like structure arranged in either parallel or as clusters. The average height of microvilli in the normal control group was found to be $8.34 \pm 0.68 \mu\text{m}$ (Fig. 1B). In chickens treated with only Cd (T1), atrophy and erosion of villi were observed and the villous tips showed bulbous shape (Fig. 1C). These changes in villi might be due to the direct effect of Cd on villi. Upon examination under higher magnification, the microvilli structure was either damaged or height of microvilli was shortened. The average height of microvilli in the Cd-exposed group was $4.89 \pm 0.90 \mu\text{m}$ (Fig. 1D) which was significantly lesser ($p < 0.05$) than all other groups. Similarity, Noda *et al.* (1978) reported that Cd treatment leads to a reduction in height of microvilli at the intestinal mucosa of rats³⁶. In vitamin C and E along with Cd-exposed group (T2), the number of villi folds and the texture of villi was found comparable to that observed in the normal control group (Fig. 1E). The average height of microvilli (Fig. 1F) in this group ($8.57 \pm 0.75 \mu\text{m}$) was significantly more ($p < 0.05$) compared to Cd-exposed group (T1) which indicates a protective effect of vitamin C and E on alterations in the structure of villi in the intestine caused by Cd in chickens. Yoo *et al.* (2016) also reported that Vitamin C and E have a role in maintaining intestinal morphology in chickens³⁷.

Table 7 — Cadmium level ($\mu\text{g}/\text{gm}$ tissue) in tissues from chickens of different treatment groups

Name of organ	C1	T1	T2	T3	T4
Kidney	0.07 ± 0.05^a	187.33 ± 32.78^c	207.83 ± 26.56^c	0.19 ± 0.03^a	114.67 ± 24.81^b
Liver	0.02 ± 0.02^a	67.17 ± 11.65^b	44.50 ± 4.87^b	0.03 ± 0.02^a	55.50 ± 12.91^b
Muscle	0.13 ± 0.02^a	2.53 ± 0.26^c	2.62 ± 0.27^c	0.11 ± 0.01^a	1.08 ± 0.09^b

Values with different superscripts in each row are significantly different ($p < 0.05$.)

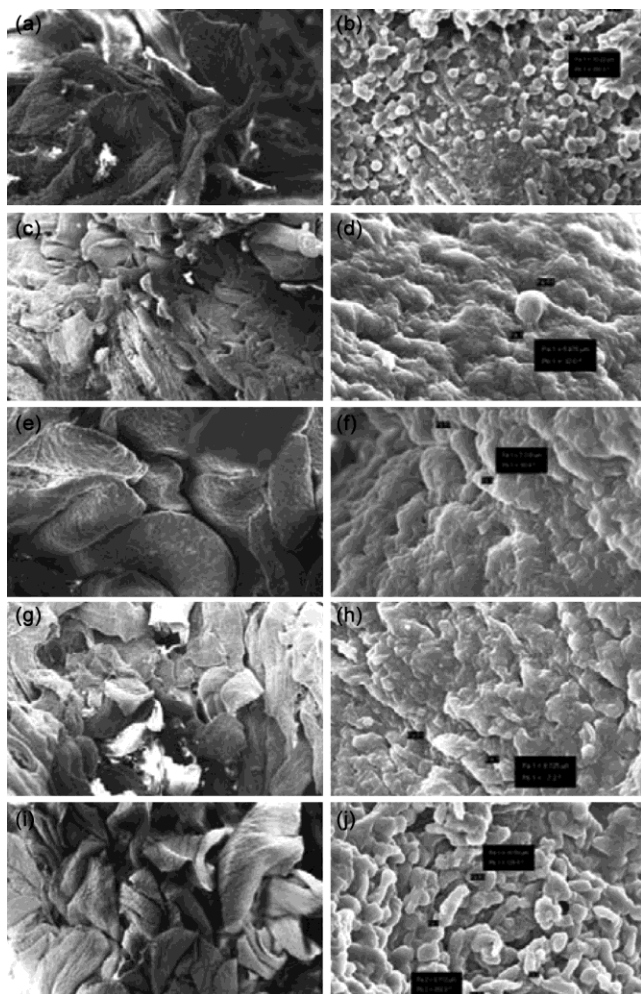


Fig. 1 — Scanning Electron Microscopy of ileum part of the intestine of chickens under various treatments; 1A & 1B- Villi and microvilli structures of C1 group, 1C & 1D- Villi and microvilli structures of T1 group, 1E & 1F- Villi and microvilli structures of T2 group, 1G & 1H- Villi and microvilli structures of T3 group, 1I & 1J- Villi and microvilli structures of T4 group

In PHMEF treated group (T3), the villous structures were found normal with clear clusters of microvilli (Fig. 1G and Fig. 1H). The average height of microvilli in this group was $9.36 \pm 0.70 \mu\text{m}$ which was significantly ($p < 0.05$) higher than that observed in Cd-exposed (T1) group. In the group treated with PHMEF along with Cd (T4), tongue-like villi with continuous intact surface were observed which were comparable to those of normal control group (C1). Upon higher magnification corrugated surface showed the absence of discontinuity and disruption of the epithelial mucosa as well as microvilli were prominent. The average height of microvilli in this group was $9.52 \pm 0.33 \mu\text{m}$ which was significantly higher than the Cd-exposed group (Fig. 1I and

Fig. 1J). The presence of many different types of phytochemicals (flavonoids, phenolics, alkaloid, saponin etc.) in polyherbal mixture might be responsible for such effect on the surface of intestine against damaging property of Cd.

Histopathological changes in kidney, liver and intestine of chickens under different groups are shown in Supplementary Fig. S1 to Fig. S3. The histopathological changes in the intestine of chickens exposed to Cd (T1) revealed degenerative changes in villi and goblet cells as well glands along with damage to muscularis mucosae. While degenerative changes in villi and goblet cells in the intestine of chickens treated with either vitamins or PHMEF along with Cd were lesser or mild compared to those observed in toxicity group (Supplementary Fig. S1). The histopathological changes in the liver of chickens exposed to Cd (T1) revealed hepatic degeneration and disturbed architecture of hepatic lobules. Degenerative changes in hepatocytes in the liver of chickens treated with either vitamins or PHMEF along with Cd were less as compared to those observed in toxicity group (Supplementary Fig. S2). The histopathological changes in the kidney of chickens exposed to Cd (T1) revealed swelling and degeneration of glomeruli, proximal and distal convoluted tubules. While less degeneration of tubules with interstitial infiltration of inflammatory cells in the kidney of group T2 was observed. The microscopic examination of the kidney of chickens treated with PHMEF along with Cd (T4) showed only mild degeneration of proximal and distal convoluted tubule (Supplementary Fig S3). Protection of kidney by PHMEF was not remarkable which might be due to the accumulation of Cd in the kidney as compared to other sites of the body.

LCQ-ToF-MS analysis of a hydroalcoholic extract of PHM showed the presence of about fifty-eight primary as well as secondary metabolites (Fig. 2). Out of all detected primary and secondary metabolites, important ones were flavonoids (like quercetin, luteolin etc.), phenolic compounds like (bergenin, Hydroxycoumarin, 3, 4-Dicaffeoyl-1, 5-quinolactone etc.), saponins and alkaloids etc. as mentioned in Table 8. In the analysis, various active principles of medicinal plants used in PHM have detected i.e., bergenin (*P. pterocarpum* leaves), solasodine (*S. xanthocarpum*), various withanolides (*W. somnifera*) etc. Other important compounds detected are Quercetin 3-glucuronide-7-glucoside, 8-Hydroxyluteolin 8-glucoside, Luteolin 7-rhamnosyl

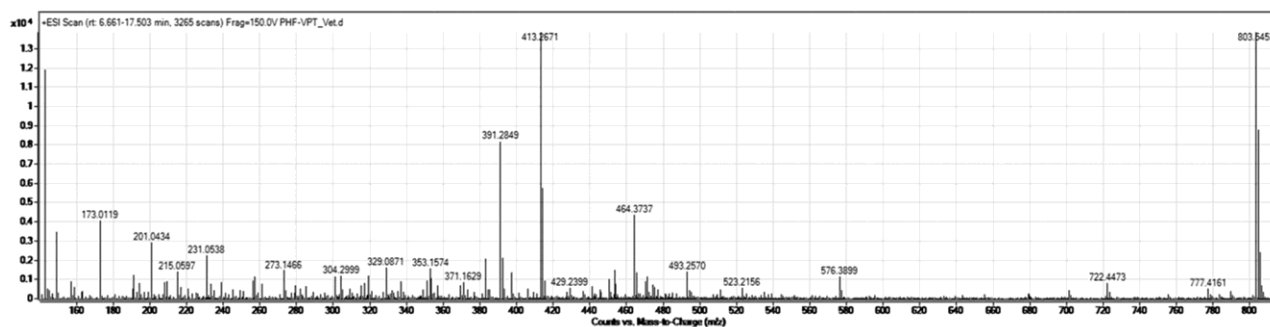


Fig. 2 — Mass spectra of LC-QTOF-MS analysis of hydroalcoholic extract of PHM

Table 8 — Major antioxidant compounds identified from polyherbal mixture using LC-QToF-MS analysis

Sr. No.	Name of compound	Formula	Score	Mass	Retention Time	Height	Area	% area	PubChem CID	CAS No.	METLIN ID	Error (ppm)
1	Bergenin	C ₁₄ H ₁₆ O ₉	99.29	328.0798	7.439	117469	1895944	7.72	66065	477-90-7	43945	1.11
2	gamma2-Solamarine	C ₃₉ H ₆₃ NO ₁₁	98.84	721.4395	12.547	34674	196344	0.80	3479482	11034-34-7	86227	-0.85
3	Arjunolic acid	C ₃₀ H ₄₈ O ₅	98.67	488.3503	14.045	35929	318958	1.30	73641	465-00-9	89977	0.26
4	3,4-Dicaffeoyl-1,5-quinolactone	C ₂₅ H ₂₂ O ₁₁	98.67	498.1156	11.096	10601	49826	0.20	102177109	-	86162	-1.23
5	Eriojaposide A	C ₂₄ H ₃₈ O ₁₁	98.47	502.2408	13.627	9249	98041	0.40	9892275	290308-51-9	92803	-1.22
6	Leptinidine	C ₂₇ H ₄₃ NO ₂	98.45	413.3287	13.371	20636	121050	0.49	185580	24884-17-1	41810	-1.64
7	Etioline	C ₂₇ H ₄₃ NO ₂	98.2	413.33	12.487	159945	968426	3.94	12309791	29271-49-6	41809	1.50
8	(14alpha,17beta,20S,22R)-14,20-Epoxy-17-hydroxy-1-oxowitha-3,5,24-trienolide	C ₂₈ H ₃₆ O ₅	98.19	452.256	12.956	14786	84941	0.35	73699089	-	88624	-0.61
9	Solamargine	C ₄₅ H ₇₃ NO ₁₅	97.91	867.4994	12.547	715082	3652131	14.86	73611	20311-51-7	68605	1.59
10	Solasonine	C ₄₅ H ₇₃ NO ₁₆	97.85	883.4941	12.487	277403	1277645	5.20	119247	19121-58-5	68609	1.31
11	Quercetin 3-glucuronide-7-glucoside	C ₂₇ H ₂₈ O ₁₈	97.63	640.1269	10.465	12425	73402	0.30	44259245	-	50617	-1.05
12	alpha-Solamarine	C ₄₅ H ₇₃ NO ₁₆	97.62	883.4942	12.487	273736	1256078	5.11	70680623	20318-30-3	89874	1.43
13	Minabeolide-5	C ₂₈ H ₃₈ O ₅	97.37	454.2723	13.823	43716	308219	1.25	52931502	-	84273	0.83
14	Lucidumol A	C ₃₀ H ₄₈ O ₄	97.1	472.3549	15.152	19560	105762	0.43	475410	217476-73-8	89100	-0.76
15	8-Hydroxyluteolin 8-glucoside	C ₂₁ H ₂₀ O ₁₂	96.5	464.0956	11.169	29650	110750	0.45	44258591	27686-36-8	49796	0.26
16	Luteolin 7-rhamnosyl (1->6)galactoside	C ₂₇ H ₃₀ O ₁₅	96.4	594.1579	11.67	8284	39621	0.16	44258135	160698-21-5	49141	-0.97
17	Pubesanolide	C ₂₈ H ₄₂ O ₅	96.29	458.3025	13.089	10299	41691	0.17	44249449	98569-64-3	89500	-1.58
18	(S)-Nerolidol 3-O-[alpha-L-Rhamnopyranosyl-(1->4)-alpha-L-rhamnopyranosyl-(1->2)-beta-D-glucopyranoside]	C ₃₃ H ₅₆ O ₁₄	96.13	676.3663	14.736	7844	37782	0.15	14060453	143376-48-1	95303	-1.05
19	Quercetin 3,5-digalactoside	C ₂₇ H ₃₀ O ₁₇	94.45	626.1471	10.737	7786	43837	0.18	44259101	-	50467	-1.92
20	Saponin H	C ₃₆ H ₅₈ O ₁₀	90.69	650.4018	13.195	6520	38837	0.16	101634402	85202-26-2	90881	-1.85
21	Coagulin R 3-glucoside	C ₃₄ H ₄₈ O ₁₁	90.42	632.32	12.547	28341	159242	0.65	73208739	221695-66-5	89077	0.53
22	3-O-(Glcβ1-2Glcβ1-4Galβ)-(25R)-12-oxo-5alpha-spirostan-3beta-ol	C ₄₆ H ₇₆ O ₁₉	90.07	932.4964	12.378	3816	14864	0.06	52931471	-	84230	-1.81
23	3-Hydroxycoumarin	C ₉ H ₆ O ₃	86.99	162.0315	8.429	11846	169345	0.69	13650	939-19-5	44078	-1.20

(1->6) galactoside. Bergenin is a C-glucoside of 4-O-methyl gallic acid found in *Peltophorum pterocarpum*³⁸. Bergenin possesses various pharmacological actions like anti-inflammatory, antioxidant, antimicrobial and anticancer³⁹. Bergenin

has been reported to have reversal effect on the elevated level of MDA and modulates the action of SOD and CAT in cyclophosphamide-induced immunosuppression and therefore reduces the injury caused by the oxidative stress⁴⁰. Solasodine is an

aglycon part of steroidal glycoalkaloid solamargine. It is commonly found in various species of *Solanum* but principally present in *Solanum xanthocarpum*⁴¹. Solasodine is capable of normalizing various antioxidant parameters like SOD, CAT, GSH and MDA. Solasodine has been found with marked reducing effect on cerebral infarction and oxidative stress and enhanced the defence mechanisms in the ischemic rats⁴². Arjunolic acid, a triterpenoid saponin, is also reported in some *Syzygium* species⁴³. The role of arjunolic acid in arsenic-induced toxicity has been studied well in terms of cardiac protection⁴⁴, hepatic, testicular and nephroprotection. It is already established that arjunolic acid postulates its action through modulation of oxidative stress at a cellular level⁴⁵. Quercetin is a flavonol and antioxidant compound. It has been isolated from leaves of *Peltophorum pterocarpum*⁴⁶ and *Cressa cretica*⁴⁷. Quercetin can reverse the oxidative damage induces by Cd chloride⁴⁸. Quercetin along with *Opuntia elatior* fruit juice also showed a preventive effect against streptozotocin-induced alterations in haematological, biochemical as well as pathological damage in major organs⁴⁹. Luteolin is a flavone type flavonoid and the main constituent of various food plants. Luteolin is also found in *Peltophorum pterocarpum* leaves⁵⁰. Luteolin exerts its action on lipid peroxidation via reduction of malondialdehyde by-product. Apart from this, luteolin also balanced the oxidative marker enzymes like SOD, CAT, GSHPx etc in isoproterenol-induced myocardial infarction in rats⁵¹.

Conclusions

Exposure of broiler chickens to Cd through drinking water at 100 ppm level for 28 days produced oxidative stress-mediated alterations. Polyherbal mixture-enriched feed (2%) comprising of equal proportion of powders of fruits of *Opuntia elatior* Mill., leaves of *Peltophorum pterocarpum* (DC) Baker ex DC, leaves of *Syzygium cumini* (L.) Skeels, aerial part of *Withania somnifera* (L.) Dunal, fruits of *Sphaeranthus indicus* (L.), leaves of *Cressa cretica* (L.), aerial part of *Solanum xanthocarpum* Schrad. & Wendl showed antioxidant effect against cadmium due to the presence of flavonoids as well as affect the accumulation of cadmium. Further study by incorporating the herbal active principles-incorporated feed with large number of birds can be planned to get more scientific information.

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Declaration of interest

None

Author Contributions

UDP: Conceptualization, Supervision, Writing - review & editing

PRB: Formal analysis, Investigation, Writing Draft

KBP: Investigation, Data analysis

HBP: Investigation, Methodology, Editing

CMM: Investigation, Methodology

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