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Characterization and Safety Assessment of Herbal Formulation (CSW/AKS/2010) in Wistar Rats: A Possible Ameliorating Remedy against Mycotoxicosis

Gauri A. Chandratre[1*], A.G. Telang[2], Prarabdh C. Badgujar[3], Sachin S. Raut[4] and A.K. Sharma[5]

[1]Department of Veterinary Pathology, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana 125004, India
[2]Toxicology laboratory, Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India
[3]Food Toxicology Section, Department of Food Science and Technology, National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonipat, Haryana 131 028, India
[4]Animal facility, CSIR- Institute of Microcrobial Technology (IMTECH), Chandigarh-160036, India
[5]Mycotic and Mycotoxic Diseases Laboratory, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India
**Corresponding author*

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A B S T R A C T

CSW/AKS/2010 is a novel herbal formulation consisting of *Withania somnifera* and *Curcuma longa* along with toxin binder yeast- *Saccharomyces cereviciae*, intended to be incorporated in livestock feed for prevention and treatment of mycotoxicoses. The present study evaluated potential safety of CSW/AKS/2010 following acute and subacute exposure in Wistar rats. Phytochemical analysis of *Withania somnifera* and *Curcuma longa* was carried out with the help of HPLC and HPTLC respectively. In acute toxicity study, single dose of 1500, 3000 and 5000 mg/kg body weight was administered by oral gavage in Wistar rats to determine its oral LD_{50}. In subacute toxicity study, herbal formulation was fed at the dose of 5 g/kg, 10 g/kg and 15 g/kg of feed for 28 days to male Wistar rats. Hemato- biochemical parameters and pathomorphological changes in vital organs were evaluated at the end of the experiment. The HPLC fingerprint of the extract showed a spectrum profile characteristic of withanolides and HPTLC chromatogram showed high concentration of curcumin. The oral LD_{50} of the present formulation was estimated to be 5000 mg/kg b.w. Body weight gain was normal in treated rats and no adverse effects were obseverd. Following subacte (28 day repeated exposure), there were no significant alterations in the above mentioned parameters between treatment and control groups. The treated animals exhibited normal feed intake and dose dependent enhancing effect on growth. CSW/AKS/2010 herbal formulation could be considered safe. No observed adverse effect level of the herbal formulation was 15 g/kg of feed.

Introduction

Globally, mycotoxins have significant human and animal health, economic and international trade implications (29, 2, 27, and 28). The biological reactions following ingestion of

one or a combination of mycotoxins vary from acute to overt disease with high morbidity and death to chronic, insidious disorders with reduced animal productivity [(2)]. Mycotoxin contamination levels in animal feedstuffs are usually not high enough to cause an overt disease but may result in economical loss through clinically obscure changes in growth, production and immunosuppression [(6)]. While many synthetic chemicals and drugs are continuously being used for the treatment of mycotoxicoses, effective measures for the prevention of mycotoxins/fungal growth are still lagging. The obvious problems with synthetic chemicals used for prevention of mycotoxins are tissue residues, reduced feed intake and nutrient utilization. However, medicinal plants do not have these concerns owing to their effects as bio-enhancer, low toxicity, less side effects and compatibility to metabolic processes. Due to the increasing cost of livestock maintenance, stressful transit in seeking professional veterinary care and the introduction of new technologies in the production of plant based therapeutics; there is increased demand in the development and assessment of herbal formulations for various diseases, conditions/ailments and beneficial health effects in animal healthcare sector [(9)].

Considering these facts, a novel herbal formulation (CSW/AKS/2010) was developed with two herbs along with yeast which could be used to prevent or counteract toxic effects of mycotoxins. The formulation used in the present study comprised of crude powders of medicinal plants possessing well characterized pharmacological activities like *Withania somnifera* [(4)], *Curcuma longa* [(23,11)] and toxin binder yeast *Saccharomyces cereviciae* [(14, 25, 16)]. Historically, both the plants have been extensively used either individually or in combination with others for variety of conditions both in human and veterinary medicine. There have been ample of evidence of use of these plants and their excellent pharmacological activities in an experimental set up; it was difficult to find documented evidence of use of these plants in veterinary ethnopharmacology. Nonetheless, farmers and tribal people have been using roots or powder of the *Withania somnifera* as a galactogouge, hepato-protectant, anti-stress, antioxidant agent and turmeric powder (from *Curcuma longa)* as an immunomodulator, anti-inflammatory and would healing agent in livestock healthcare.

Although herbal entities are believed to be relatively safe in humans and animals, toxicity characteristic needs to be confirmed prior to their evaluation in targeted species. Generally, this is accomplished by conducting general preclinical safety studies to uncover potential toxic effects of drug in question [(19)]. With the above considerations, the present study was aimed to evaluate the acute and subacute (repeated dose, 28 days) toxicity of novel herbal formulation developed at our laboratory in Wistar rats. We hypothesized that this formulation could be specifically used as an agent for the prevention and/or amelioration of mycotoxicoses.

Materials and Methods

Preparation of herbal formulation

Herbal formulation prepared in our laboratory consisted two medicinal plants- *Withania somnifera*, *Curcuma longa* and a mycotoxin binder - yeast *Saccharomyces cereviciae*, in powder form. Dried roots of *Withania somnifera* and rhizomes of *curcuma longa*, were procured from local market and duly authenticated. A voucher specimen number 2012038104b and 2012030760b for *Withania somnifera* and *Curcuma longa* respectively were deposited in herbarium of Department of Botany, Bareilly College, Bareilly, India. Mycotoxin binder was procured from Lyka

Healthcare Ltd., Mumbai, India. The plant materials were ground and made in fine powdered form. Three powders were mixed together in appropriate proportion to obtain the herbal formulation CSW/AKS/2010 (under Intellectual Property). All the components used in the formulation were tested for the presence of mycotoxins (Aflatoxin B1, Ochratoxin and T-2 toxin) residues by high performance thin layer chromatography method (HPTLC).

Phyto-chemical analysis of plant extracts

Qualitative analysis of extracts

The air dried roots of *Withania somnifera* and rhizomes of *Curcuma longa* were finely powdered using a cutter mill and extracted using methanol and distilled water separately. 1 g of sample powders were dissolved in 30 ml methanol and water each separately, incubated overnight at room temperature, shaked on rotary shaker, and filtered through Whatman filter paper number 1. Filtrate was evaporated to dryness. Respective extracts were re-dissolved in 5 ml methanol and distilled water separately. Phytochemical analysis of aqueous and methanolic extracts of *Withania somnifera* and *Curcuma longa* was carried out using standard qualitative method as described previously [7, 26]. The phytochemicals detected were tannins, anthraquinones, coumarins, proteins, flavonoids, trierpenes, sterols, saponins, glycosides and reducing sugars.

Quantitative analysis of extracts

Withania somnifera

Extraction

Extraction of withanolides from the root powder of *Withania somnifera* was carried out in four volumes of 50% methanol followed by de-fattening and de-pigmentation three times with an equal volume of n-hexane and recovering withanolides from the defatted methanolic extract into chloroform by liquid – liquid partitioning as described earlier [21]. Chloroform extract was evaporated to dryness at 25°C and re-dissolved in known volume of methanol. The aliquot was filtered through 0.22µm nylon filter. 20µl was injected into HPLC system for analysis.

Quantification of withanolides in extract

Quantification of withanolide A, withaferin A and other withanolides was carried out using an HPLC method as described [22]. Separation was achieved by using RP-C_{18} column (Phenomenex, particle size 5µm 4.6 X 250mm) as stationary phase. The mobile phase consisted of mixture of water (A) and methanol (B), each containing 0.1% acetic acid (Gradient A:B, 60:40 to 25:75, 0 to 40 min; 10:90, 40 to 50 min) at a flow rate of 0.6 ml/min.

The column temperature was set at 27°C and withanolides were detected at 227nm wavelength (PDA dectector). Authentic standards of different withanolides (Natural Remedies Pvt. Ltd., Bangalore, India) were used for preparing the calibration curves and their concentration in *Withania somnifera* root extract was determined from the respective peak area.

Curcuma longa

Extraction

The air dried rhizomes of *Curcuma longa* were fine powdered using a cutter mill and extracted using methanol. 1 g of sample powder was dissolved in 30 mL methanol, incubated overnight at room temperature, shaked on rotary shaker, filtered and evaporated to dryness. The residue was re-

dissolved in 1 mL of methanol and samples of varying volume (µl) were spotted for quantification.

Quantification of curcumin in extract

HPTLC was carried out on aluminium backed silica gel $G_{60}F_{254}$ plates (Merck, Germany) for detection of curcumin in extract. Methanolic solution of extract and curcumin standard of known concentrations were applied to the thin layer chromatography (TLC) plate as 6 mm wide bands positioned 15 mm from the bottom and 15 mm from the side of the plate, using a CAMAG Linomat 5 TLC applicator with the nitrogen flow providing a delivery speed of 150 nL/s from the syringe. These parameters were kept constant throughout the analysis of all the samples. Plates were developed in a CAMAG twin trough glass tank pre-saturated with the mobile phase, Benzene: chloroform: methanol (15:80:5, v/v) for one hour. Quantitative analysis of the curcumin in the extract was done by scanning the plate using TLC scanner equipped with Wincats software (CAMAG) applying the following conditions: slit width- 6 x 0.45 mm, wavelength (λmax)- 254 nm, and absorption-scan mode. The identification of curcumin was confirmed by superimposing the UV spectra of samples and standards within the same R_f window. Authentic standard of curcumin (Sigma Aldrich, India) was used for preparing the calibration curves and its concentration in *curcuma longa* rhizome extract was determined from the respective peak area.

Experimental animals

Young male Wistar rats (6-8 weeks of age) weighing 80-90 g were obtained from Laboratory Animal Resources (LAR) section of Indian Veterinary Research Institute, Izatnagar and housed in a room maintained under standard laboratory conditions of controlled temperature (22 ± 3 °C) and humidity (45 ± 15%) with 12 h light/dark cycle. Rats were randomly assigned in polystyrene cages (6 rats per cage) with free access to basal feed (procured from the Feed Processing Unit of the Institute, tested negative for the presence of possible residual mycotoxins- AFB1, OTA and T-2), water and were acclimatized for one week prior to the start of experiments. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) as per the rules and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Acute oral toxicity study

To assess the acute oral toxicity of CSW/AKS/2010, it was administered as a suspension in 0.5% aqueous carboxymethyl cellulose (CMC) to six male rats at 1500, 3000 and 5000 mg/kg body weight (in a volume of 5 ml/kg b.w.) as a single oral dose. The rats were fasted overnight before and 3 h after the administration of the test material and only water was provided during the period. All the animals were closely observed for mortality and clinical signs for first 4 h after dosing, periodically during first 24 h and thereafter twice daily for 14 days consecutively. The body weight of rats was recorded on day 0, 7th and 14th day [20]. Rats were anaesthetized with isoflurane and necropsy was carried out at the end of the experiment.

Sub-acute toxicity study

This study was conducted as per the OECD guideline 407 (OECD, 2008) with minimum modifications. Twenty four male Wistar rats were randomly divided into four groups. One group served as control and was provided basal feed and remaining groups were fed basal feed mixed with herbal formulation at

three graded dose levels viz: 5 g/kg, 10 g/kg and 15 g/kg of feed. The experimental feed was prepared on weekly basis and provided *ad lib* for 28 days. Dosage selection of herbal formulation was based on three factors, viz., the dose used in the acute toxicity study, safe dose limit of each component in herbal formulation and daily feed intake of each rat (15-25 g/day).

Clinical signs and general behavior

Before treatment, rats were individually handled and carefully examined for abnormal behavior and appearance. During treatment period all rats were observed at least twice in a day for clinical signs and detailed observations were recorded on weekly interval.

The observations included but were not limited to: general appearance (skin, fur, changes in eyes and mucous membranes), body position and posture (i.e. hunchback posture), autonomic nervous system function (lacrimation, respiration, excretion, piloerection), motor coordination, ambulatory abnormalities (e.g. changes in gait were assessed weekly by allowing the rat to walk freely), reaction to being handled and to environmental stimulation, neurobehavioral effects (tremor, convulsion, muscular contractions), changes in exploratory behavior, ordinary behivor (changes in grooming, head shaking, gyration), abnormal behavior (autophagia, backward motion, abnormal vocalization), and aggression. Individual body weight data were obtained at weekly interval during treatment period.

Absolute and relative organ weight

At the end of the experimental trial, rats were sacrificed and liver, testes, kidneys, spleen, thymus and brain were excised, lightly blotted on tissue paper and weighed; all data were expressed as absolute and relative organ weight.

Hematology and Biochemical Parameters

Hematological and Biochemical Parameters were performed at weekly interval in present study. Blood samples were collected from retro-orbital plexus of each rat for hematological and serum biochemical analysis. Following hematological parameters were determined using Vet Scan HM5 auto-hematology analyzer (ABAXIS, USA): Total Erythrocyte Count (TEC), Total leukocyte count (TLC), packed cell volume (PCV), Haemoglobin (Hb) and Total Thrombocyte count. Blood smears were prepared immediately and stained with Giemsa stain to examine the morphology of cells.

Blood samples were collected without anticoagulant, allowed to clot to separate serum for biochemical analysis viz. Total protein (Biuret method), alanine amino transferase (ALT), aspartate amino transferase (AST), and creatinine (alkaline picrate method), using standard kits (Span Diagnostics Ltd., Surat, India) employing GENESYS 10 UV spectrophotometer (Thermo Electron Corporation, Madison, Wisconsin, USA).

Pathomorphological studies

Gross pathology

At the end of the exposure period, rats were euthanized by deep inhalation anaesthesia and systematic necropsy examination was conducted. All the internal organs were thoroughly examined and the gross lesions, if any, were recorded.

The criteria of gross pathological examination were based upon the position, shape, size, color and consistency of the organs.

Histopathology

Small representative pieces from various organs/tissues were collected from each animal and fixed in 10% neutral buffered formalin.

After proper fixation (48 h), tissues were processed conventionally to obtain 4-5µm thick haematoxylin and eosin (H & E) stained sections for histopathological evaluation.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) followed by Tukeys test to evaluate significant difference between groups.

The criterion for statistical significance was set at $p < 0.05$. All experiments were repeated at least twice.

Results and Discussion

Phytochemical analysis of plant extracts

Qualitative phytochemical Analysis of plant extracts

Aqueus and methanolic extracts for roots and rhizomes of *Withania somnifera* and *curcuma longa* respectively exhibited minor differences in the presence of phytochemical groups and the results were compared in the Table-1.

Phytochemical screening indicated that roots of *Withania somnifera* contained tannin, coumarins, proteins, triterpenes, sterols, resins and reducing sugar; whereas rhizomes of *Curcuma longa* showed presence of tannin, flavonoids, triterpenes, reducing sugar and glycosides.

Quantitative phytochemical analysis of plant extracts

Withania somnifera

HPLC chromatogram of withanolides standard and withania somnifera root extract (I and II respectively) has been shown in Fig.1A. The concentration of withaferin A and withanolide A in WS root extract by HPLC method was found to be 60 mg/100gm and 20 mg/100gm respectively.

Curcuma longa

The HPTLC densitogram for simultaneous display of *Curcuma longa* rhizome extract samples and curcumin-standard (I and II respectively) are depicted in Fig. 1B. Characterization of the methanolic extract of *Curcuma longa* rhizome extract by HPTLC confirmed the presence of curcumin, and its content was found to be 5.516 µg/g of rhizome powder.

Acute toxicity study

No mortality was observed after administration of herbal formulation in any of the test doses during 14 days period. No behavioral changes were observed during the observation period. The test formulation did not reveal any adverse effect on body weight gain compared to control rats at 5000 mg/kg b.w. (data not shown). The oral LD_{50} of the herbal formulation was estimated to be greater than 5000 mg/kg b.w. This experiment suggests that herbal formulation is practically non-toxic.

Subacute toxicity study

Daily feeding of herbal formulation for 28 consecutive days did not induce any obvious symptom of toxicity in rats at any of the test doses. All animals were active and healthy

throughout the period of study. No lethality was recorded during the 28 days of dietary exposure of herbal formulation. No differences in general behavior, food and water consumption were observed between the test and control groups. At the dose of 15 g/kg of herbal formulation, body weight of rats increased significantly ($p < 0.05$) from 7th day onwards; whereas feeding of 5 g/kg and 10g/kg of herbal formulation resulted in increase in body weight from 15th day onwards as compared to control ($p < 0.05$) (Table 2). Herbal formulation in three graded doses showed enhancing effect on body weight of experimental rats.

Absolute and relative organ weight

There was no significant effect on absolute as well as relative organ weights of liver, kidneys, spleen, thymus, testes and epididymis in the treated compared to control rats except that of brain weight. A marginal increase in the absolute organ weight of the brain was observed in rats fed herbal formulation at 10 g/kg and 15 g/kg of feed; however, no dose dependent effect was observed in treatment groups (Table 3 and 4).

Hematology and biochemical parameters

Feeding of herbal formulation did not cause any significant change in the hematological parameters as compared to control animals.

However, total erythrocyte count was found to be increased in all rats including that of control group on 21st day which was significantly reduced on 28th day (Table 5). In case of biochemical parameters, there was significant increase in the aspartate transaminase in all rats of treatment and control groups from 14th day onwards; whereas total protein, ALT and creatinine values did not differ significantly from control (Table 6).

Pathomorphological studies

Significant gross pathological lesions were not recorded in any of the organ at necropsy (Fig. 2). Similarly, histopathological examination did not reveal any treatment-related changes in the vital organs (Fig. 3 and 4).

The present investigation was undertaken to evaluate the safety of CSW/AKS/2010, a herbal formulation intended to be used in control and treatment of mycotoxicoses in animals. Two herbs, i.e. *Withania somnifera* and *Curcuma longa* along with a yeast *Saccharomyces cereviciae*, were incorporated in the formulation keeping in view the antioxidant, immunomodulatory, antibacterial and antifungal properties of these herbs and toxin binding properties of the yeast. Both of these herbs have been extensively studied and reported to enhance growth performance of animals as well as boost the immune system. *Saccharomyces cereviciae* has been reported to act as toxin binder [(25)] without reducing nutritive value of the feed and thus helps in prevention of deleterious effects of toxins in animals.

Phytochemical characterization of the *Withania somnifera* root powder by HPLC revealed presence of active principles, i.e. withannolides, withaferin A and withanolide A. The Ayurveda, Siddha and Chinese traditional recipes have described that, withaferin A and withanolides (alkaloids) not only counteract oxidative stress but also decreases activity of cholinesterase and xanthine oxidase [(5)]. The rhizomes of *Curcuma longa* used in the present study were also characterized by HPTLC and found to contain high concentration of curcumin. Curcumin, the major component in *Curcuma longa L.* is a non-toxic, highly effective natural antioxidant and is responsible for its wide spectrum of biological actions.

Fig.1 A. HPLC chromatogram of *Withania somnifera* root extract; I-Withanolides standard, II-Root extract. B. HPTLC chromatogram of *Curcuma longa* rhizome extract; I-Curcumin standard, II-Rhizome extract

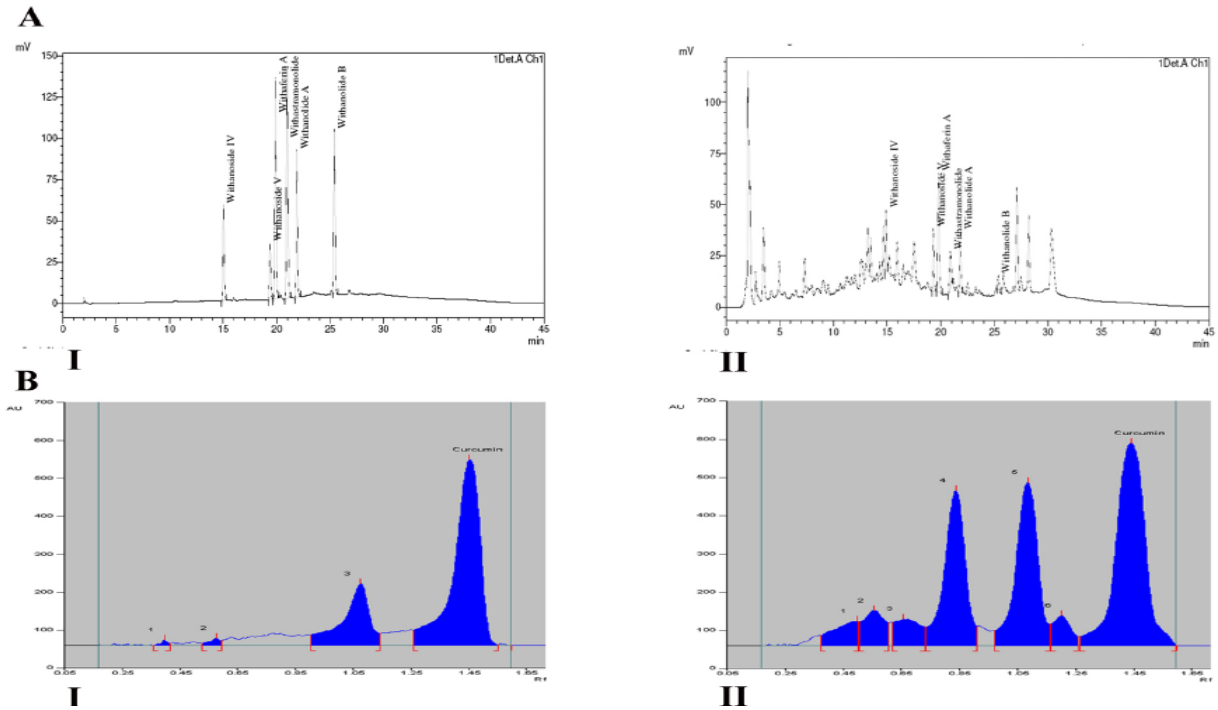


Fig.2 Effect of herbal formulation-CSW/AKS/2010 on gross pathology of vital organs – liver, spleen, kidney and thymus

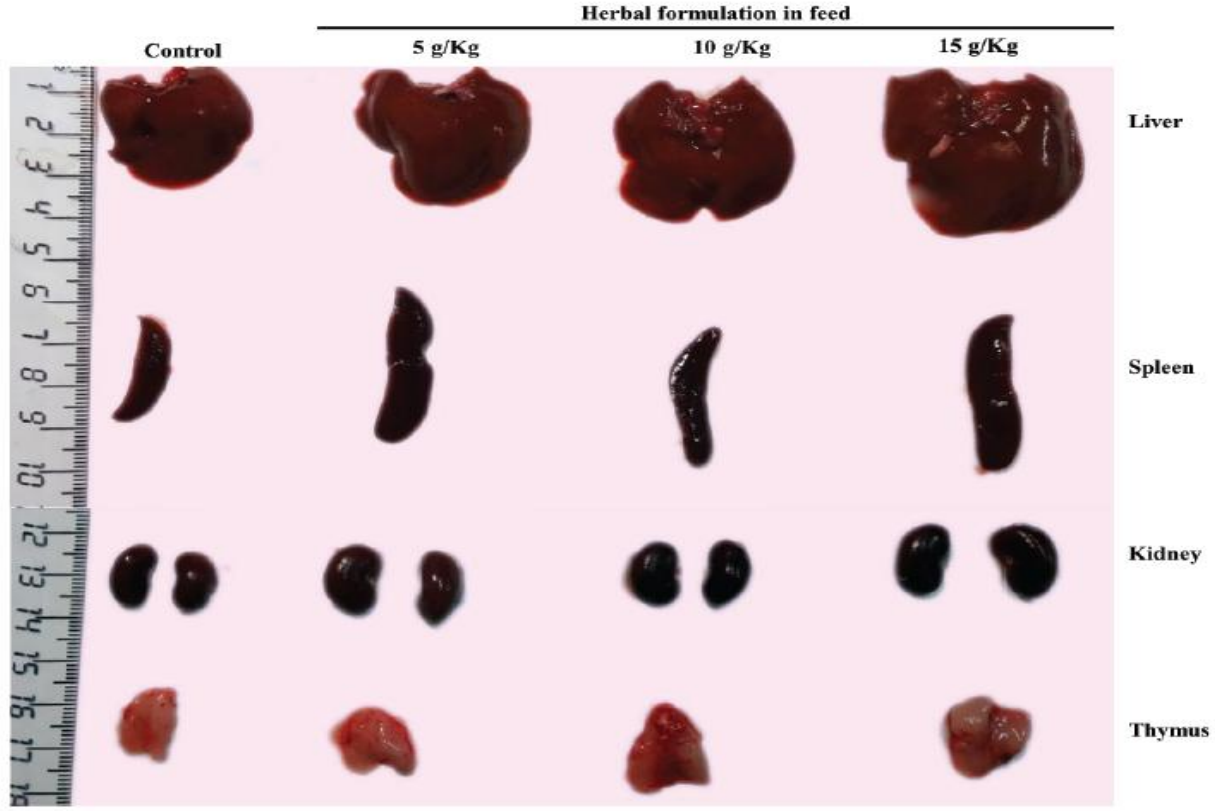


Fig.3 Representative Photomicrograph showing histo-pathological architecture of the major organs of Wistar rats fed herbal formulation-CSW/AKS/2010 at various dosage for 28 days (H & E, ×100). No abnormal pathological changes were observed. Testis: showing adequate spermatogenic activity in the seminiferous tubules; Liver: showing central vein (CV) and normal hepatic lobular pattern; Kidney: showing well preserved histological structure of glomeruli (G) and tubules (PCT, DCT); Cerebrum: showing normal appearance of layered cortex, ML-Molecular layer, OGL-Outer granular layer, PCL-Pyramidal cell layer, GL-Ganglionic layer

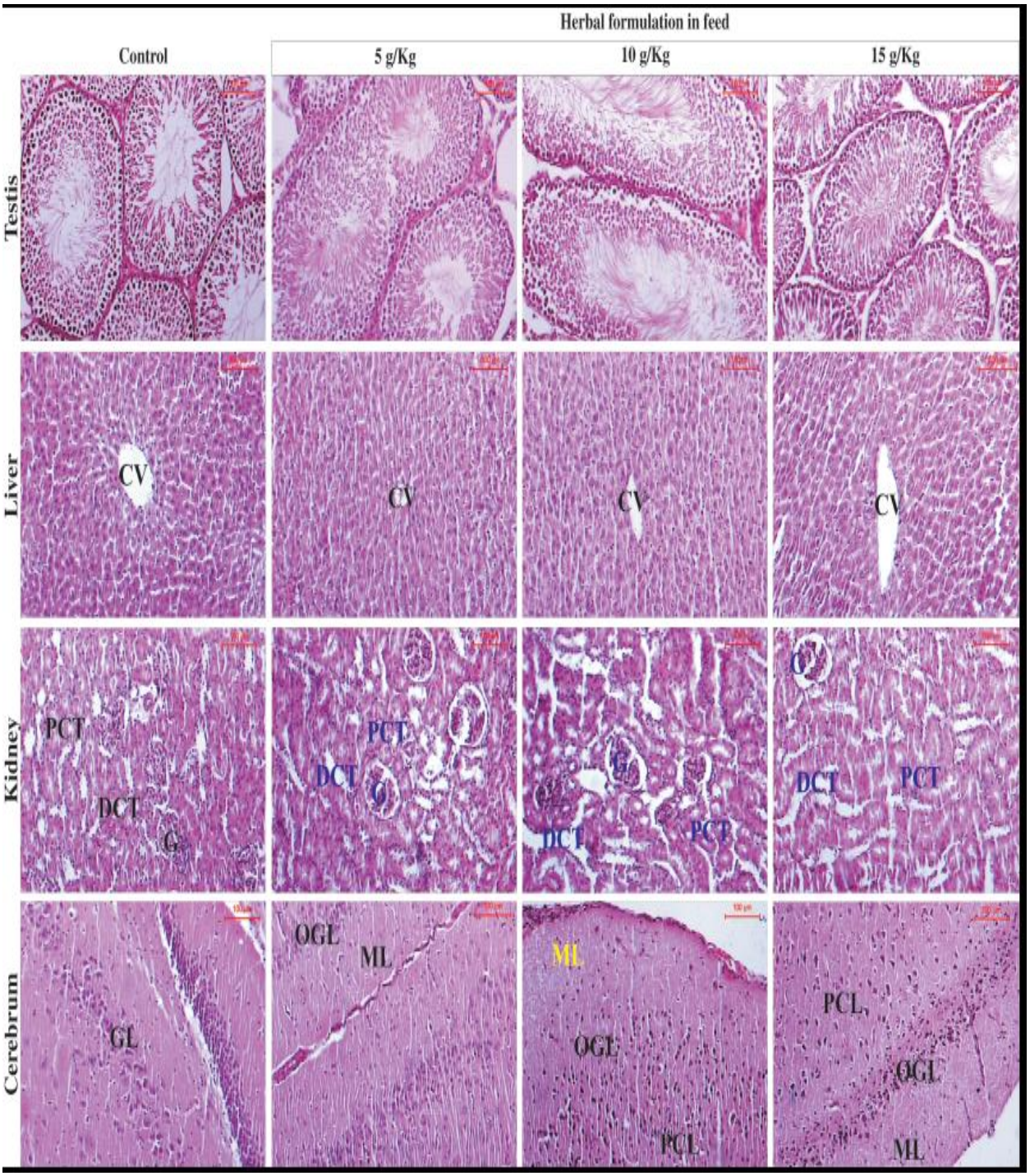
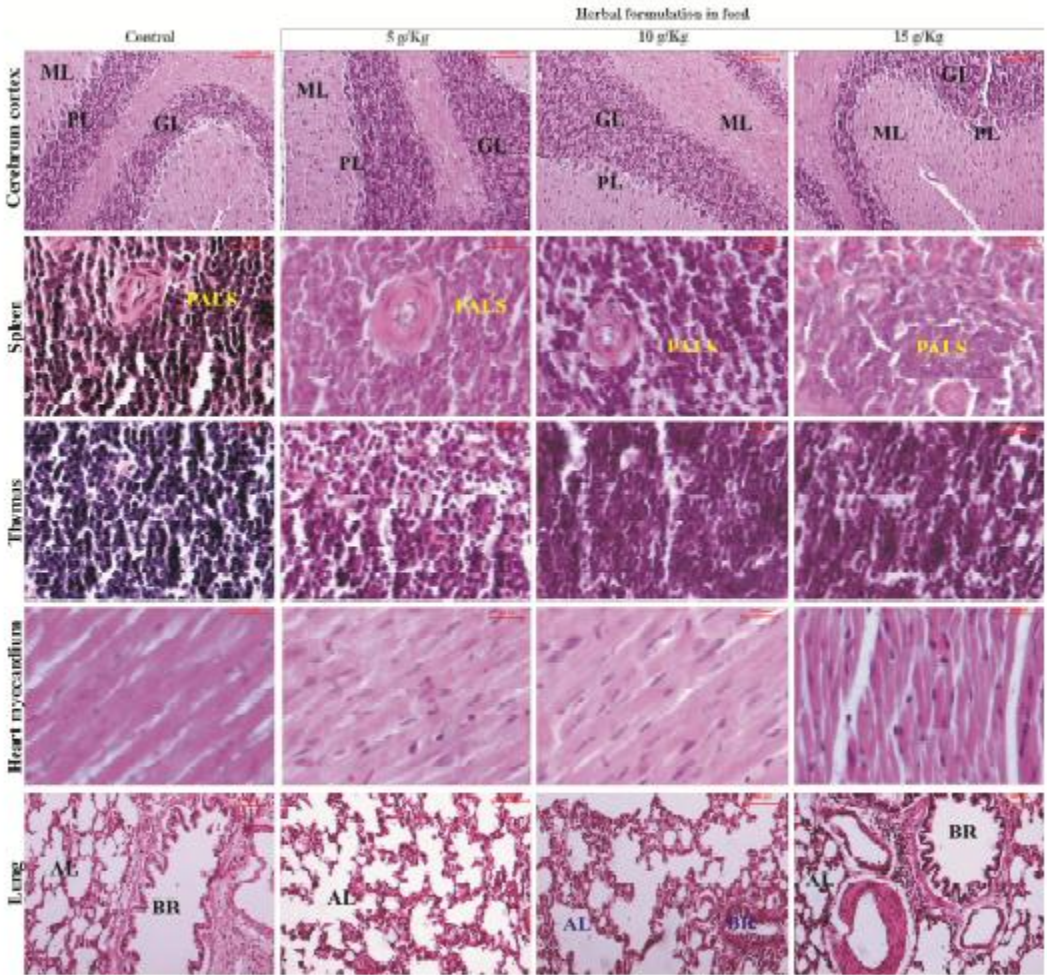


Fig.4 Representative Photomicrograph showing histo-pathological architecture of the major organs of Wistar rats fed herbal formulation-CSW/AKS/2010 at various dose rates for 28 days. No abnormal pathological changes were observed. Cerebellar cortex: showing normal appearance of three layers- ML: Molecular layer, GL: Granular layer, PL: Purkinje cell layer (H & E, ×100); Spleen: showing peri-arteriolar lymphoid sheath (PALS) containing adequate lymphocytes (H & E, ×400); Thymus: showing adequate population of lymphocytes in the cortical area (H & E, ×400); Heart myocardium: showing cardiac muscle fibers forming interconnecting network (H & E, ×400); Lung: showing normal appearance of bronchiole (BR) and alveoli (AL) in terminal portion of respiratory tree (H & E, ×400)



The safety of the individual herbs used in the present formulation has been known since ancient times and demonstrated experimentally by various researchers [1, 10, 18]. However, toxicity of combined formulation is usually unknown and need to be confirmed compared to their chemical counterparts [16]. Therefore, in the present study acute and sub-acute toxicity of this herbal formulation was tested in Wistar rats. In the acute oral toxicity study no deaths were recorded upto the dose of 5000 mg/kg body weight. Since, substances with an LD_{50} values from 5000 to 15,000 mg/kg body weight are classified as practically non-toxic [13, 20]; our formulation could be considered as practically non-toxic. Typically, subacute oral toxicity studies are carried out to evaluate the likely adverse effects upon prolonged exposure of a test substance in animals to gather information

about deleterious health effects of repeated exposures including target organ toxicity, cumulative effects and to determine the dose level at which there is no observed adverse effect [17].

In the subacute toxicity study, it was observed that test formulation was well tolerated upto 15 g/kg of feed. Absence of treatment related deaths or toxic signs throughout the study were direct indications of relatively harmless nature of the test compound over prolonged exposure. The compounds with toxicity potential are believed to have impact on feed intake, metabolic processes and consequently on body weight gain. A decrease of more than 10% in body weight gain is considered to be detrimental on long term administration of test material (8, 24). Such trend was not observed in the current study. The body weight gain of different groups remained comparable till the end of the study period with corresponding normal feed consumption clearly demonstrated normal metabolic processes in rats fed with herbal formulation. Rather, it was observed that the body weight gain of the animals in formulation treated groups increased in dose and duration dependent manner.

Functional observation tests such as open field observations, locomotor activity, grasping strength of treated rats revealed no considerable alterations. These results in general demonstrate that the herbal preparation did not interfere with neuromuscular physiology and autonomic activity of treated animals. Although some of the hematological and biochemical parameters (TEC, TTC and AST) were found to be significantly increased or decreased as compared to control group, the values were within the normal reference ranges specified for the species [12]. Since no consistent dose dependent changes were observed in blood parameters such variation could be considered spontaneous, incidental and non-treatment related in test species.

Repeated dose safety studies provide information on target organ toxicity upon continuous exposure of test substance intended for prolonged use in target species [17]. The present investigation did not record any treatment related gross pathological lesions. A statistically significant difference was observed in the absolute weight of brain in treatment groups which could be due the individual animal variation. Neither dose dependent effects nor any significant change were recorded in the absolute and relative weight of other vital organs in all the treatment groups. The normal values of majority of hematological and clinical chemistry end points did not reveal organ damage related effects. With respect to target organ toxicity, no significant test compound related histopathological alterations were found in the vital organs of treated rats (Fig. 3 and 4).

In conclusion, the present study demonstrated that oral lethal dose of this formulation exceeds 5000 mg/kg body weight. Feeding of CSW/AKS/2010 for 28 days did not produce any detrimental effects on various biological parameters and was found to be safe in rats. No observed adverse effect level (NOAEL) of formulation could be considered as 15 g/kg of feed in rats. Moreover, components of the formulation reported in our study are readily available, economical and can be easily prepared, mixed in the feed by livestock owners. Nevertheless, its efficacy as well as possible ameliorating potential against mycotoxins needs to be thoroughly evaluated.

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