

Assessment of sub-chronic, hematological and histopathological toxicities of a herbal combination

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Abstract: The herbal combination under study consists of *Withania somnifera*, *Tribulus terrestris*, *Mucuna pruriens* and *Argyria speciosa*. Present study is mainly designed to investigate the gross physical, sub-chronic, hematological and histopathological effects of the combination widely used for its stimulating, revitalizing and fertility boosting effects in Pakistan. Sub-chronic, hematological and histopathological outcomes of herbal combination were assessed on 27 albino rabbits weighing from 1000gm-1500gm after giving herbal combination for 60 days in two doses 27 and 81mg/kg against control. No significant toxicity was revealed during the entire period of study, however some biochemical changes were observed in kidney and liver but these changes did not coincide with histopathological findings. There was no mortality and evidence of systemic toxicity including hematological toxicity following 60 days administration of herbal combination. Results of present study suggest that further studies are required on large number of animals before reaching to a definite conclusion, more over clinical studies should also be conducted to confirm the possible toxic effects of the herbal combination.

Keywords: Herbal combination, sub chronic toxicity, hematology and histopathological toxicity.

INTRODUCTION

Plants have been used for their medicinal value for thousands of years. Approximately 25% of our current prescription drugs are plant-based. These products have been used as medication and supplements all over the world especially in remote and urban areas of developing countries (Khan *et al*, 2013). Traditional and herbal medicines are still the most popular remedy all over the world for the treatment of various ailments and its use is encouraged at governmental level *e.g.* in China it is practiced along with western medicine at every level of healthcare (Hesketh and Xing, 1997; Zhang *et al*, 2011).

Globally, herbal medicines have been researched under rigorous controls and approved by the governments of various countries, however most developing countries lack proper legislation and guidelines for the proper preparation and usage of herbal drugs. In developed countries, herbal drugs are generally treated as dietary supplement and separate guidelines have been made by World Health Organization about the assessment and safe use of these drugs (WHO, 1991; 2005).

Herbal drugs have great potential for improving health and lowering health care cost, these drugs relatively appears safe but little preclinical or clinical work has been done ensure the efficacy and safety of these products, hence extensive consumption of herbal medicines justify the evaluation of inherent safety of these products.

Herbal combination under study consist of *Withania somnifera*, *Tribulus terrestris*, *Mucuna pruriens* and *Argyria speciosa*, which, has been used for several years due to its bio-stimulating and fertility enhancing effects (Gauthman *et al*, 2002; Riaz *et al*, 2010).

Withania somnifera is of great therapeutic value in the indigenous medical systems for more than 3000 years. Large doses may cause gastrointestinal upset, diarrhea and vomiting (Qamaruddin *et al*, 2012; Morgan *et al*, 2014).

It has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent and more lately to treat bronchitis, asthma, ulcers, insomnia, and senile dementia (Bone, 1996). Clinical and preclinical studies support the use of *W. somnifera* for anxiety, cognitive and neurological disorders, inflammation, and Parkinson's disease (Archana *et al*, 1999; Bhattacharya *et al*, 2002; Qamaruddin *et al*, 2012).

W. somnifera mainly contains steroidal alkaloids and lactones in a group of constituents called withanolides, which has been held responsible for much of its pharmacological activity. The two cores withanolides are withaferin A and withanolide D.

T. terrestris is primarily found in nutritional supplements marketed to enhance testosterone levels in athletes. Historically product-containing Tribulus has been used as all-purpose tonic and treatment for impotence (McKay, 2004). *T. terrestris* is also recommended in cases of

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spermatorrhea (Rowland and Tai, 2003) and urinary disease e.g. dysuria, chronic cystitis, gonorrhoea and painful micturition (Joshi *et al*, 2001; Bhandari *et al*, 2013).

The active ingredient in Tribulus are thought to be furostanol saponins, although no significant side effects are predictable at doses of Tribulus present in commercial nutritional supplements, but animal studies have suggested the possibility of locomotor disturbances at high doses of Tribulus (Aslani *et al*, 2003).

M. pruriens has been used in various disorders of nervous system due to its high concentration of L-dopa in the seeds and has been investigated for its probable use in Parkinson's disease. *M. pruriens* is rich in protein, fiber, fat, ash, carbohydrates, albumin, valine, tryptophan, cysteine, methionine, leucine, oleic, linoleic and palmitic acid (Sidduraju *et al*, 1996; Kavita and Thangamani, 2014).

Plant extract of *M. pruriens* has shown marked hypotensive and hypocholesterolemic effects in normal rats (Joshi, 2000). *M. pruriens* seed produced hypoglycemic and hypocholesterolemic effects in normal rats (Pant *et al*, 1968). However, such diet had insignificant effect in alloxan-treated rats (Jophi and Pant, 1970). Another study had shown that protein isolated from the seeds reduce cholesterol content of the liver and blood in rats (Kavita and Thangamani, 2014).

Indole alkyl amines isolated from various parts of *M. pruriens* have shown marked behavioral changes including excitation, tremors, piloerection. They antagonized pentobarbitone-induced hypnosis, inhibited reserpine-induced ptosis, showed hypothermia and sedation, reduced chlorpromazine-induced catatonia and increased the amphetamine toxicity in rats (Bhattacharya *et al*, 1969; Szabo, 2003).

Roots and seeds of *A. speciosa* are commonly used parts and contains tannin, resin and alkaloids e.g. chanoclavine, ergine, ergonovine, and isoergine. It has been used extensively used as anti-inflammatory, anti-arthritis, immuno-modulatory, aphrodisiac, antiphlogistic, antiseptic, tonic and emollient (Gokhale *et al*, 2003; Joseph *et al*, 2011).

Hence present study has been conducted to investigate the detailed toxicological effects of the herbal combination, since it has been used for its revitalizing and fertility enhancing effects without any prescription from a licensed or trained medical practitioner.

MATERIAL AND METHOD

Selection of animals

Present investigation was done on 27 healthy white rabbits of either sex weighing from 1000-1500 grams; all

animals were housed individual cages, under controlled condition of temperature (23±2°C) and humidity (50-60%). Diet and water was provided *ad libitum*.

Experimental design

All animals were equally divided into three groups (9 animals / group) one served as control while, other two as treated groups. Before giving drug physical health of these animals was checked during the conditioning period under the laboratory environment for a period of 7 days specially noticing loss of hair, diarrhea, edema, ulceration and lack of activity.

Herbal combination was given in two doses 27 and 81 mg/kg for a period of 60 days orally. Dose was prepared in DMSO 500mg/5ml. Control group was given DMSO through oral route equivalent to the volume of respective doses.

Sample collection

7ml blood sample was collected from each animal by cardiac puncture at the completion of dosing period of 60 days.

Assessment of toxicities

Physical examination

The gross examination of animals was carried out at every week following administration of herbal combination precisely noticing average weight variation, loss of hairs, skin ulceration, loss of interest in food, loss of activity, hematuria, lacrimation, salivation, vomiting, diarrhea, edema, muscle tone, tremor, and aggressive behavior. Autopsy was performed by random selection, at the completion of dosage and blood sample collection for biochemical tests.

Biochemical evaluation

Blood samples were immediately centrifuged (Heraeus, Christ Labofuge A) at 4000 rpm for 8 minutes to collect serum, which were then analyzed within 3 hours on Vita Lab eclipse automatic analyzer (Merck) using standard reagent kits supplied by Merck.

Hematological examination

Hematological parameters e.g. RBC, WBC, Platelet and hemoglobin were measured on Humacount plus automatic hematology analyzer from Human Germany.

Histopathological examination

Specimens of heart, liver and kidney were conserved in 10% buffered formalin till further processing at Dr. Ehsanullah Lab (Private) Ltd. suitable blocks of these organs were taken, fixed and the sections were cut for microscopic examination.

STATISTICAL ANALYSIS

All readings were mentioned as mean and standard error to the mean and analyzed using one way unstacked

ANOVA, p values were observed (Larson and David, 1992). Results were taken significant if p value was less than 0.05 and highly significant if less than 0.005.

Cardiac parameters

Creatine Kinase (CK-NAC)

The concentration of CK-NAC in serum was determined photometrically using improved standard method conferring to the recommendations of the German Society of Clinical Chemistry, using standard kits from Merck.

CK-NAC reduces NADP to NADPH, which is directly proportional to the CK-NAC activity in the sample. The decrease in absorbance was measured at 340 nm, every minute for 3 minutes on Vitalab that directly gives the concentration of the enzyme CK-NAC in U/l.

Lactate dehydrogenase (LDH)

The concentration of enzyme LDH in serum was determined photometrically using standard kits from Merck. This method is based on reduction of NAD to NADH by LDH which is specifically detected by colorimetric assay and directly proportional to the LDH activity in the sample.

The decrease in absorbance was measured at 340 nm, every minute for 3 minutes on Vitalab that directly gives the concentration of the enzyme LDH in U/l.

Aspartate transaminase (AST)

The concentration of enzyme AST in serum was determined with the help of "Ecoline 125, ASAT supplied by Merck. The rate of NADH consumption was measured photometrically, which is directly proportional to the AST activity in the sample.

The decrease in absorbance was measured at 340 nm, every minute for 3 minutes on Vitalab that directly gives the concentration of the enzyme AST in U/l.

Renal parameters

Total protein

Serum protein was determined according to biuret method using Vitalab Eclipse in which proteins and peptides produce a violet color complex with copper ions in presence of sodium hydroxide (Thomas, 1998). Sample absorbance was checked against reagent blank at 546 nm within 60 min. on Vitalab that directly gives the total protein concentration in gm/dl

Urea

Urea is determined enzymatically. The decrease in NADH absorbance per unit time is proportional to the Urea concentration. Absorbance was measured at 340 nm in Vitalab that directly gives the urea levels in mg/dl

Creatinine

Serum creatinine activity was determined by a colorimetric reaction called Jaffe reaction (Seeling and Wuest 1969). Creatinine forms a yellow- orange

compound in alkaline solution with picric acid. The concentration of the dye over a certain reaction time is a measure of the creatinine concentration (Bartels *et al*, 1972). Absorbance of sample and standard were read at 492 nm on Vitalab that directly gives serum creatinine activity in mg/dl

Uric acid

Uric acid was determined using Uricase method by Vitalab Eclipse (Barham and Trinder, 1972). Uricase oxidizes uric acid to Allantoin. The generated peroxide reacts with 4-aminoantipyrine and 2, 4, 6-tribromo-3-hydroxybenzoic acid (TBHBA) to form Quinoneimine. Absorbance of a sample was read against the reagent blank within 60 min, using Vitalab Eclipse that directly gives the Uric acid.

Hepatic parameters

Alkaline phosphatase

The concentration of enzyme alkaline phosphate in serum was determined photometrically, with the help of Ecoline 125 AP supplied by Merck. The rate of increase in 4-nitrophenolate was determined photometrically, which was directly proportional to the activity of enzyme alkaline phosphatase present in sample serum (Deutsche, 1972). The increase in absorbance was measured at 405nm every minute for 3 min, on Vitalab, that directly gives the concentration of enzyme alkaline phosphatase in U/l.

Alanine transaminase (ALT)

The concentration of enzyme ALT in serum was determined using Ecoline 125, ALAT Tris of Merck. The rate of NADH utilization was measured photometrically, which was directly proportional to the ALT activity in the sample. The decrease in absorbance was measured at 340 nm every minute for 3 minute, on Vitalab that directly gives the concentration of the enzyme in U/l.

γGT

The activity of enzyme, γ -GT in serum was determined using Ecoline S+, gamma-GT of Merck based on Szasz Method. The increase in the rate of formation of 5- amino -2-nitrobenzoate was determined photometrically, which was directly proportional to the activity of enzyme, γ -GT in the sample serum (Szasz, 1976). The increase in absorbance was measured at 405 nm, every minute for 3 minute on Vitalab that directly gives the enzyme concentration in the sample.

Total bilirubin

Total bilirubin concentration in serum was measured by Jendrassik and Grof (1938) method coupling diazotized sulfanilic acid after the addition of Caffeine, Sodium benzoate and Sodium acetate. A blue azobilirubin is formed in alkaline Fehling solution II. The absorbance of sample was measured at 578 against the blank, on Vitalab that directly gives the total bilirubin concentration in the sample in terms of mg/dl.

Table 1: Effects of herbal combination on biochemical parameters after 60 days

Parameters	Animal groups		
	Control	Herbal Combination 27 mg/kg	Herbal Combination 81 mg/kg
CK-NAC U/I	601±79	548±75	*354±41
LDH U/I	442±101	485±81	*269±60
AST U/I	52.60±5.50	58.9 0±8.10	48.80±13
Total Protein gm/dl	6.53±0.47	7.41±1.10	7.02±0.67
Urea mg/dl	33.3±3.60	37.6 0±4.00	44.2±5.20
Creatinine mg/dl	0.78±0.17	0.71±0.15	0.95±0.16
Uric Acid mg/dl	1.56±0.51	1.23±0.41	0.78±0.21
Alkaline Phosphatase U/I	74.80±16	62.90±12	79.8 0±18
SGPT U/I	80.20±5.6	112.10±22	75.70±12
ΓGT U/I	7.44±1.00	9.33±2.10	7.78±0.92
Tot. Bilirubin mg/dl	0.27±0.04	0.38±0.07	0.36±0.05
Dir. Bilirubin mg/dl	0.10±0.02	0.13±0.02	0.17±0.04
Cholesterol mg/dl	26.80±4.10	*46.60±8.30	28.60±4.50
Triglycerides mg/dl	51.70±6.50	43.7 0±3.90	50.6 0±8.90

n=9, Average values ± S.E.M, *p<0.05 significant as compared to control

Table 2: Effects of herbal combination on calcium, phosphorus, glucose and hematological parameters after 60 DAYS

Parameters	Animal groups		
	Control	Herbal Combination 27 mg/kg	Herbal Combination 81 mg/kg
Calcium mg/dl	12.64±1.50	11.57±1.20	15.19±1.90
Phosphorus mg/dl	7.48±1.60	7.94±0.82	9.12±1.70
Glucose mg/dl	127 .00±6.9	137.30±30	112.80±10
Hemoglobin gm/dl	9.34±0.69	9.4±0.69	9.27±0.63
RBC x10 ⁶ / μl	5.25±0.40	5.21±0.45	5.06±0.31
WBC x10 ³ / μl	4.66±0.55	4.33±0.63	4.52±0.70
Platelet x10 ³ / μl	404.00±87	319.4 0±31	225.60±24

n=9, Average values ± S.E.M

Direct bilirubin

The direct bilirubin is measured as the red azo dye at 546 nm using the method of Schellong and Wende (1960) without the addition of alkali. The absorbance of sample was read at 546 nm against blank, on Vitalab that gives direct bilirubin estimation of sample serum in mg/dl.

Lipids

Triglycerides

Triglycerides are enzymatically hydrolyzed to glycerol and free fatty acids by lipases. The H₂O₂ formed will be transferred to a colored Chinonimine by a peroxidase-catalyzed reaction with 4-Aminoantipyrine and Chlorophenol (Fossati and Prencipe, 1982; McGowan *et al*, 1983). The absorbance of sample and standard (200mg/dl) was measured at 500nm against the reagent blank on Vitalab.

Cholesterol

Cholesterol was measured photometrically by CHOD-PAP method, using Vitalab Eclipse. The colorimetric indicator is quinonimine, which is generated from 4-

Aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder’s Reaction).

The absorbance of sample and standard (200 mg/dl) was measured within 60 minutes, at 546nm against the reagent blank on Vitalab that directly gives the concentration of Cholesterol in mg/dl

Blood glucose

The serum concentration of glucose was determined through its enzymatic oxidation by glucose oxidase. The colorimetric indicator quinonimine was generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase. The absorbance of sample and standard (100 mg/ml) was measured against the blank within 60 minutes at 546nm.

Calcium

Calcium was determined with Cresolphthalein complexone (CPC) using Vitalab. CPC interacts with Calcium ions in alkaline medium developing a red-violet color. Interference by Magnesium is eliminated by

addition of 8-Hydroxyquinoline. The absorbance of sample and standard (10 mg/dl) was read absorbance at 578 nm against reagent blank on Vitalab Eclipse that directly gives the concentration of Calcium.

Phosphorus

Phosphate was determined using Ammonium molybdate which results in formation of inorganic phosphorus molybdate complex. The absorbance of sample and standard (5 mg/dl) was read against blank within 60 min at 340 nm on Vitalab Eclipse that directly gives the concentration of phosphorus.

Hematological estimation

Blood samples were collected under 10% EDTA at 7.2 pH and Hemoglobin concentration, RBCs, WBCs and platelet counts were measured on Humacount plus a fully automated hematology analyzer (Human Germany).

Histopathological evaluation

Representative blocks from different areas of heart, liver, and kidney were cut from each sample after separating all fat from respective organs. The blocks were processed in an automatic tissue processor (Gilford 101 system).

Tissue sections of 3-4 micron thickness were taken from the wax blocks by rotary manual microtome. The tissue sections were mounted on slides and were dried gently by pressing with filter paper. The mounted slides were placed initially for drying on a hot plate (45°C) for 90 minutes and then left in an incubator at 37°C overnight to dry.

RESULTS

Physical examination

Animals in any group did not reveal any significant toxicities and gross anomalies during the total period of experimentation i.e. 60 days. There was no skin ulceration, hematuria, loss of hair, loss of activity, vomiting, diarrhea, edema, salivation, tremor and aggressive behavior. The data also reveals no significant difference in the average weight variation.

Biochemical evaluation

Table 1 shows the effect of herbal combination following its oral administration in two doses i.e. 27 and 81 mg/kg against control on Cardiac enzymes e.g. CK-NAC, LDH & AST, Renal parameters like Total Protein, Urea, Creatinine and Uric acid, Hepatic parameters e.g. Alkaline Phosphates, ALT, γ GT, Total and Direct Bilirubin and Lipids.

There was significant decrease in the serum levels of CK-NAC and LDH and significant increase in the levels of cholesterol in the animals received herbal combination at the dose of 81 mg/kg and 27mg/kg respectively as compared to control, while changes in all other parameters were insignificant as compared to control.

Hematological parameters

Table 2 shows the effect of herbal combination following its administration at 27 and 81 mg/kg on Calcium, Phosphorus, Glucose and Hematological parameters. There were no significant changes in any of the parameters at any dose as compared to control.

Histopathological examination

Gross examination of various vital organs e.g. liver, heart, and kidney did not disclose any macroscopic changes in any group, similarly there were no microscopic changes in the heart (Plate 1) and liver (Plate 2) of animals treated with herbal combination for 60 days, however animals treated with herbal combination at 27 mg/kg revealed mild chronic inflammation in kidney (Plate 3), while animals of control group also revealed chronic interstitial inflammation in kidney (Plate 4).

DISCUSSION

Herbs have been used since earliest times as remedies for the management of variety of diseases, and despite excessive advances in modern medicine in recent years plants are still making a major contribution to health care. According to the World Health Organization, about 80% of the world's population in developing countries mainly depends on plants for prime health care (WHO, 2005).

It is proposed that nearly 25% of all recent medicines are directly or indirectly obtained from higher plants (WHO, 2005; Kunle *et al*, 2012). Chief pharmaceutical companies have shown repeated curiosity in exploring higher plants as a source for new lead molecules and also for the growth of standardized phytotherapeutic agents with proved efficacy, safety and quality (De smet, 1997; Blumenthal, 1999).

Herbal medicinal preparations are generally popular in developing countries with a long history of use and their use is also common in some developed countries e.g. Germany, France, Italy and the United States where appropriate guidelines for registration of such medicines exist (Linh *et al*, 2013).

All medicinal agents cause substantial unpredicted effects including toxicity and herbals medicines are no different. There are few reports that describes side effects caused by these agents (Dobbins and Saul, 2000; Barnes *et al*, 2007; Tsai *et al*, 2012). Hence increasing consumption of herbal medicines justifies the assessment of inherent safety of these products; even studies continue to show their efficacy.

Thus current study was design to assess sub chronic toxicity toxicological effects of an herbal combination by assessing toxic potential on gross behavioral activity and biochemical, hematological and histopathological parameters.

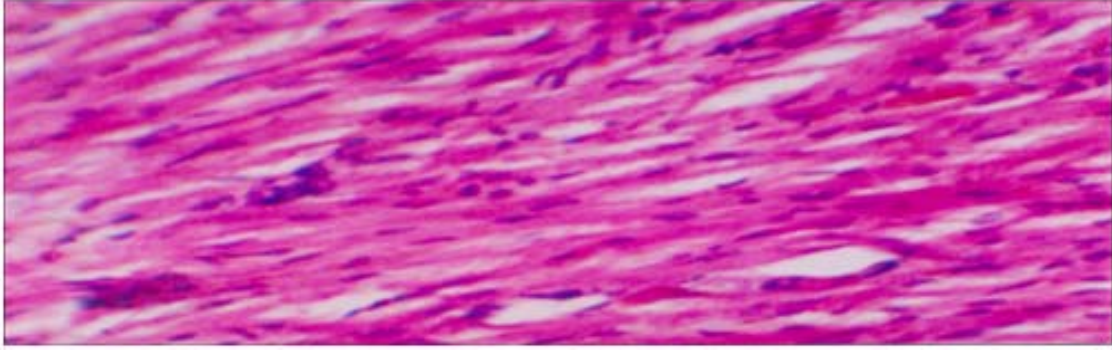


Plate 1: Cardiac issue showing no microscopic changes.

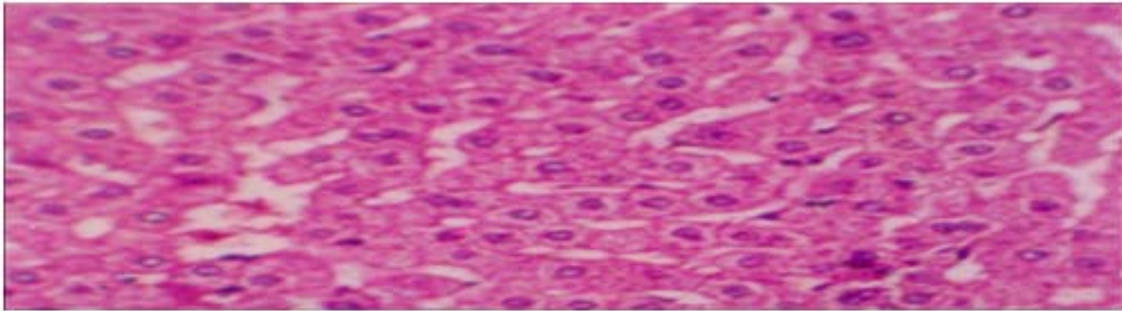


Plate 2: Hepatic tissue showing no microscopic changes.

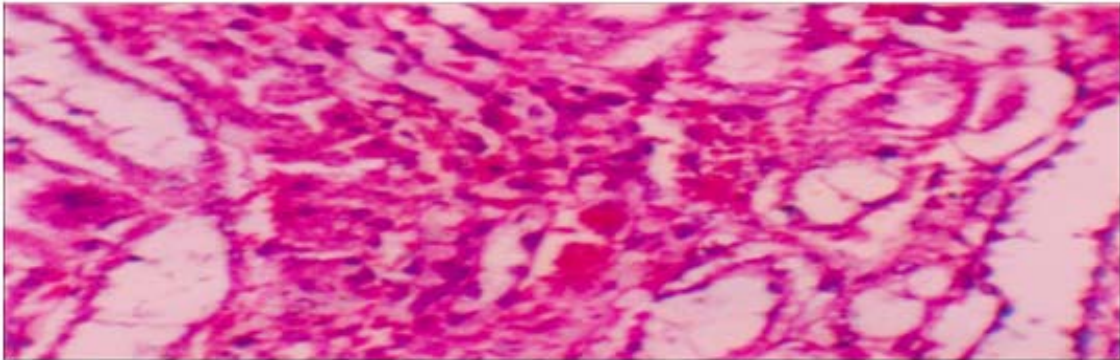


Plate 3: Renal tissue showing mild chronic inflammation.

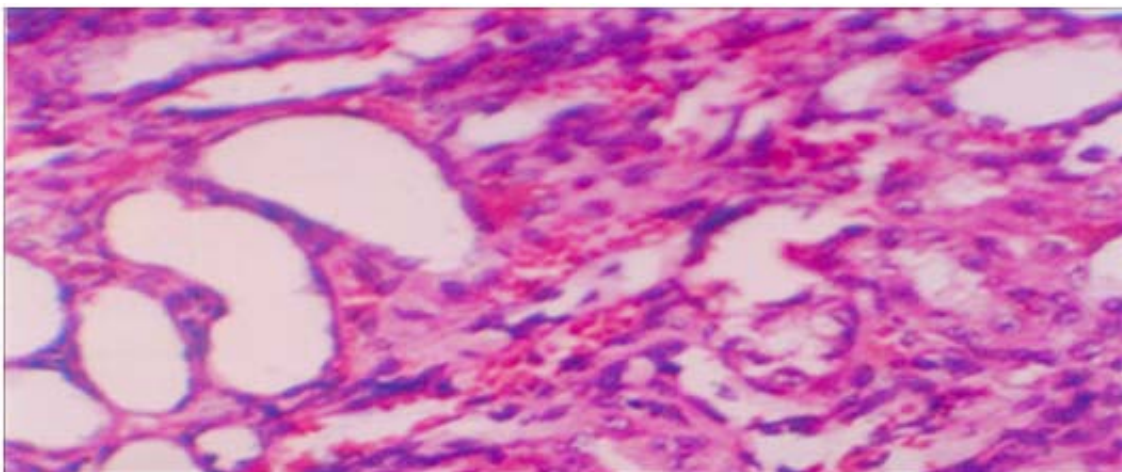


Plate 4: Renal tissue showing chronic interstitial inflammation.

Drug under investigation was administered at two different doses to rabbits for 60 days, however no gross behavioral toxicity was observed including weight variation, nor any animal died during the entire period of study.

Herbal combination was well tolerated at both doses and showed no macroscopic changes, while microscopic examination of vital tissues in animals of both groups also did not reveal any remarkable change in liver and heart however there was mild chronic inflammatory response in kidney of one animal at normal dose, while chronic inflammatory changes were also observed in animals of control group, which shows that these changes might be due to some housekeeping issues or due to intra individual variation.

Results of the hematological parameters at both doses also shows no remarkable changes in comparisons to control group, similarly there were also no significant changes in glucose, calcium, phosphorus, cholesterol and triglycerides levels as compared to control. Overall comparison of biochemical parameters and histopathological examination did not reveal any significant correlation between biochemical and histopathological findings suggesting that these changes are due to intra-individual variations and therefore are not very important.

On the basis of these findings it may be concluded that herbal combination under investigation lacks significant toxic effects and appears to be safe however further studies on large number of animals and humans are essentially required to confirm these effects.

ACKNOWLEDGEMENT

Authors are thankful to Herbion Pakistan Limited for providing financial assistance to conduct the study.

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