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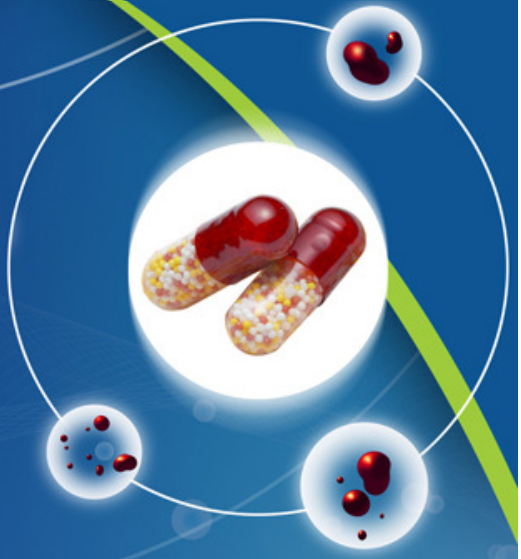

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Research Article

TO STUDY CHRONIC TOXICITY FOR POLYHERBAL FORMULATION

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ABSTRACT

The objective of the present study to investigate the safety of polyherbal formulation containing Tribulus terrestris, Withania somnifera and Chlorophytum borivilianum by determining its potential toxicity after chronic administration in rats as per OECD guideline No.421. Wistar rats (150-200g) in treatment group I, II and III received polyherbal formulation of 400 mg/kg/day, 800 mg/kg/day and 1600 mg/kg/day (p.o.) respectively for 90 days. After 90 days rats were decapitated and vital organs (brain, heart, kidney, liver, ovary, testis and epididymis) removed, weighed and made histological sections. Result showed no significant treatment-related changes in organ weights and absence of any disease state or lesions in histological sections of brain, heart, liver, kidney observed. But there were changes in weights and histoarchitectural changes (toxic effects) of ovary, testis and epididymis in group wise observed.

Keywords: Chlorophytum borivilianum, Withania somnifera, Tribulus terrestris, Chronic Toxicity Study, Wistar Rats, Histology of Vital Organs.

INTRODUCTION

Tribulus terrestris L. (Zygophyllaceae), *Withania somnifera* (Solanaceae) and *Chlorophytum borivilianum* (Liliaceae) are found widely distributed in warmer region of the world [1]. In traditional folk medicine, different plant parts like dried Fruits of *Tribulus terrestris,* dried roots of *Withania somnifera* and dried, tuberous roots of *Chlorophytum borivilianum* have been used since ancient times. *Tribulus terrestris* has been used as an aphrodisiac, anti-diabetic, anti-inflammatory, anti-pyretic, anti-stress, antiurolithiatic, hypoglycemic, hypolipidemic, revitalizing agents and in urinary infections, oedema, gout and other ailments [1-6].

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Withania somnifera has been used as an anti-aging, aphrodisiac, anti-oxidant, anti-depressant, anti-diabetic, anti-inflammatory, anti-stress, cardioprotective, hypatoprotecive, hypolipidemic, immunomodulating agent, rejuvenator [1,7-10]. *Chlorophytum borivilianum* has been used as an aphrodisiac, anti-oxidant, anti-stress antimicrobial, in arthritis, hypolipidemic, hypocholesteremic and increases body immunity, remedy for diabetes, revitalizer, general sex tonic, spermatogenic property, tonic for general debility [1,2,11-13],etc. All above mentioned three herbs have been used for their aphrodisiac activity for maximum time apart from other uses. In today's busy, hectic, stressful life many people (both male and female) use *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* in individual form or in different preparations along with other herbs like *Allium sativum, Asparagus racemosus, Crocus sativus, Ginkgo biloba, Mucuna Pruriens, Panax ginseng, Tinospora cordifolia* or *Turnera*

aphrodisiaca, etc. Because *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* increase levels of various hormones; including testosterone, estrogen and for this reason improve sports performance, productiveness in men and women, sexual functions, symptoms of menopause and vigour [1,12-19] but there are very less scientific data of toxicity available to support longer use. Use of plants as a source of medicines has been inherited and is an important component of the health care system in India. In the Indian systems of medicine, most practitioners formulate and dispense their own recipes; hence this requires proper documentation and research. In western world also, the use of herbal medicines is steadily growing with approximately 40 per cent of population reporting use of herb to treat medical illnesses within the past year [20,21]. Therefore it is necessary to study whether they have any toxic effects or not if use for longer duration of time and this study also help in selection, preparation of appropriate dose of drugs for longer duration of time. Hence the present study was undertaken to evaluate the chronic toxicity study of polyherbal formulation containing Tribulus terrestris, Withania somnifera and Chlorophytum borivilianum as per OECD guideline-421.

MATERIALS AND METHODS

Plant materials

The ethanolic extract of *Tribulus terrestris,* hydroalcoholic extract of *Withania somnifera* and hydroalcoholic extract of *Chlorophytum borivilanum* were obtained from M/S. SHAMANTAK ENTERPRISES, Pune. Certificate of authentification number of *Tribulus terrestris* is-APCP/23/2013-14, for *Withania somnifera* is APCP/30/2013-14 and for *Chlorophytum borivilianum* is APCP/24/2013-14.

Animals and housing conditions

Healthy male and female wistar rats (150-200 g) were used. They were maintained at 25 ± 2 ºC, relative humidity of 45 to 55% and under standard environmental conditions with 12:12 hr light/dark cycle in polypropylene cages. The animals were fed with standard pellet feed (Nutrivet life sciences, Pune) and water was given ad libitum. The Institutional Animal Ethics Committee approved the protocol. All the experiments were carried out between 9:00 hr to 16:00 hr.

Dosing of rats

Polyherbal formulation was freshly prepared daily combining the extracts of *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* in proportion of 1:1:1 in distilled water before dosing. In all experimental protocol, control group received vehicle (distilled water, p.o.) and three other groups received different doses of polyherbal formulation (p.o.): for group I-400 mg/kg/day), for group II-800 mg/kg/day, for group III-1600 mg/kg/day. Each group has six male and six female rats (Table I). The highest dose level had chosen with the aim of inducing toxic effects but not death or severe suffering and a descending sequence of dose levels had selected with a view to demonstrating any dosage related response and no-observed-adverse effects (NOAEL) at the lowest dose level (as per OECD guideline-421).In duration of 90 days of dosing body weights had been taken from 0 to 14th weeks.

Table I: Rats were divided in following way

Group Name	*Total Animals Required*		*Treatment*	*Duration of Treatment*
	Male	*Female*		
Control	6	6	Vehicle-10 mg/kg, p.o.(distilled water)	90 days dosing
Group I	6	6	PF-400 mg/kg, p.o.	
Group II	6	6	PF-800 mg/kg, p.o.	
Group III	6	6	PF-1600 mg/kg, p.o.	

PF-Polyherbal Formulation, n-number of animals in each group

After 90 days dosing, rats were decapitated and vital organs like brain, heart, kidney, liver, ovary, testis and epididymis were removed and weighed. Then fixed organs in 10% buffered formalin, processed routinely and stained with hematoxylin and eosin. Histological sections were prepared from middle of organs. The sections were examined with Image Microscope (Magnus, Pune) under 4X, 10X, 40X magnification values for identification of any lesions, degeneration or disease conditions.

Statistical analysis

The result of body weight and organ weight were expressed as mean ± SEM (n=6). The data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significance set at *p<0.05, **p<0.01.

RESULTS

Change in body weight and organ weight and histological analysis of the organs.

The 90 days control group

The body weight of wistar rats has increased normally (Table II, III) and no significance changes has observed in relative weights of vital organs (Table IV, V and Figure 1,2,3,4,5) like brain, heart, kidney, liver, ovary, testis and epididymis in control group.

Table II: Body weight (kg) of male wistar rats of each group

Male			
Control (distilled water- 10 mg/kg/day)	Group I (400 mg/kg/day)	Group II (800 mg/kg/day)	Group III (1600 mg/kg/day)
210± 11.07	217.66 ± 12.69	195.5 ± 3.03	197.83 ± 2.05
213.5 ± 10.94	219.83 ± 13.40	199.83 ± 3.84	203 ± 2.14
218.5 ± 10.77	231.5 ± 15.14	196.83 ±4.23	205.16 ± 2.60
221.83 ± 11.35	232.16 ± 13.47	202.5 ± 3.58	208.16 ± 2.84
224.33 ± 11.06	237.66 ± 12.58	206.5 ± 3.83	214.16 ± 5.73
229.83 ± 10.82	242.83 ±12.40	211.33 ± 2.56**	215.66 ± 6.31
233 ± 10.67	235.16 ± 9.141	209.33 ± 2.51*	212.16 ± 8.62
234 ± 10.75	238.83 ± 10.90	210.66 ± 2.18*	215.33 ± 8.76
238.16 ± 10.62	241.166± 11.36	211.5 ± 2.43**	219.5 ± 9.13
234.66 ± 10.41	241.5 ± 11.92	214.83 ± 2.45**	222.66 ± 9.05
237.33 ± 10.45	242.16 ±12.87	218.83 ± 2.45**	224.83 ± 9.78
240.16 ± 10.74	247.5 ± 13.04	222.83 ± 2.44**	228.83 ± 9.78
242.33 ± 10.08	251 ± 12.96	226 ± 2.68**	232.66 ± 9.69*
245 ± 10.007	255.16 ± 12.89	229.33 ± 2.75**	236.5 ± 9.86**
247.33 ± 9.96	260.16 ± 13.17	238 ± 5.31**	240.83 ± 9.78**

Body weight (kg) of male wistar rats of each group treated during 90 days dosing with vehicle (control group) and polyherbal formulation (treated group) (*p<0.05, **p<0.01: ANOVA - Dunnett).

Table III: Body weight (kg) of female wistar rats of each group

Female			
Control (distilled water-10 mg/kg/day)	Group I (400 mg/kg/day)	Group II (800 mg/kg/day)	Group III (1600 mg/kg/day)
209 ± 11.07	218.16 ± 13.28	196.66 ± 3.07	233.33 ± 9.60
212.5 ± 10.94	221.5 ± 13.23	200.83 ± 3.84	238 ± 9.66
216.5 ± 10.77	221.43 ± 9.72	197.5 ± 3.98	241.3 ± 9.73
220.83 ± 11.35	226.16 ± 9.81	203.9 ± 3.27	244.66 ± 9.36
225.33 ± 11.06	232.53 ± 10.16	207.38 ± 3.74	248.16 ± 9.47
228.83 ± 10.82	225.91 ± 11.48	212.11 ± 2.60	253.16 ± 9.37
232 ± 10.67	222.61 ± 9.89	216.61 ± 3.20	255.16 ± 9.26
234.83 ± 10.60	219.66 ± 12.90	220 ± 3.48*	254.33 ± 8.32
237.16 ± 10.62	210.93 ± 10.67	210.16 ± 3.44	252.83 ± 7.91
233.66 ± 10.41	217.33 ± 12.40	213.66 ± 3.32	255 ± 7.76
236.33 ± 10.45	215.31 ±11.81	200.81 ± 8.75	258.5 ± 7.80
237.66 ± 10.46	220.16 ± 12.09	211.8 ± 7.62	260.66 ± 7.79
239.16 ± 10.38	220.5 ± 11.15	215.33 ± 7.30	261.5 ± 7.37
240.16 ± 10.38	221.66 ± 10.03	219.83 ± 6.69*	263.33 ± 7.28
241.166 ± 7.85	224.66 ± 10.27	222.71 ± 6.69**	265.83 ± 7.37

Body weight (kg) of female wistar rats of each group treated during 90 days dosing with vehicle (control group) and polyherbal formulation (treated group) (*p<0.05, **p<0.01: ANOVA - Dunnett).

Table IV: Organ weight (g) of male wistar rats of each group

Sex	Organ	Control (distilled water- 10 mg/kg/day)	Group I (400 mg/kg/day)	Group II (800 mg/kg/day)	Group III (1600 mg/kg/day)
Male	*Liver*	9.96±0.13	9.86±0.10	9.75±0.11	9.8±0.09
	Brain	2.28±0.06	2.28±0.04	2.25±0.04	2.2±0.03
	Heart	1.35±0.04	1.35±0.04	1.28±0.03	1.23±0.03
	Testis	1.26±0.07	1.4±0.05	1.28±0.06	1.19±0.06
	Kidney	1.09±0.05	1.14±0.06	1.05±0.04	1.03±0.05
	Epididymis	0.46±0.02	0.51±0.05	0.46±0.02	0.43±0.02

Organ weight (g) of male wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett).

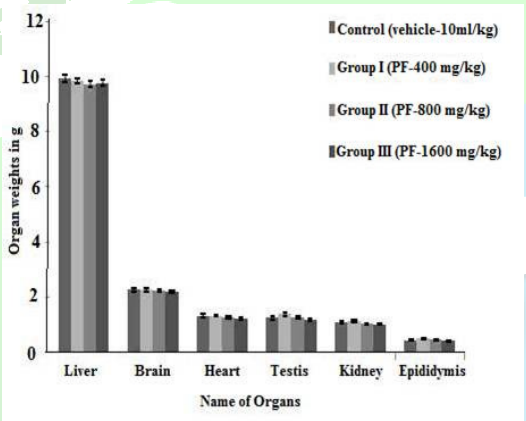


Figure 1: Organ weight of male rats of each groups

Organ weight (g) of male wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett) (Separate graphs of Testis and Epididymis are given below).

PF-Polyherbal Formualtion

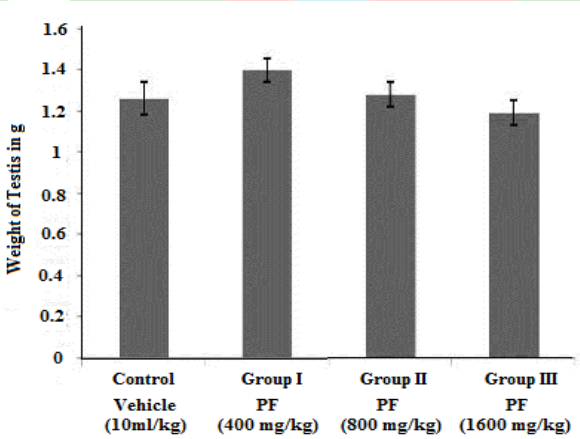


Figure 2: Weight of Testis of male rats of each group

Testis weight (g) of male wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett).

PF- Polyherbal Formulation

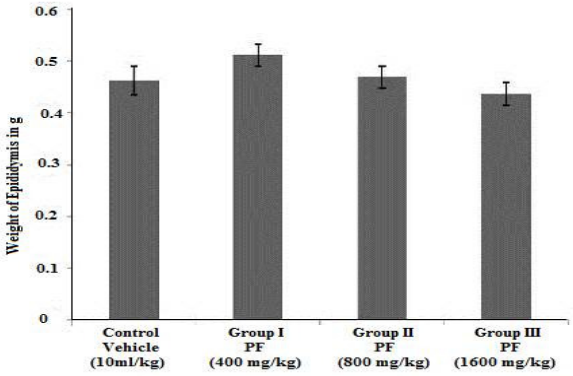


Figure 3: Weight of Epididymis of male rats of each group

Epididymis weight (g) of male wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett).

PF- Polyherbal Formulation

Table V: Organ weight (g) of female wistar rats

Sex	Organ	Control (distilled water- 10 mg/kg/day)	Group I (400 mg/kg/day)	Group II (800 mg/kg/day)	Group III (1600 mg/kg/day)
Female	*Liver*	9.78±0.11	9.76±0.10	9.68±0.08	9.8±0.09
	Brain	2.28±0.06	2.25±0.02	2.25±0.04	2.2±0.03
	Heart	1.33±0.03	1.35±0.04	1.28±0.03	1.23±0.03
	Kidney	1.1±0.06	1.18±0.06	1.10±0.06	1.06±0.50
	Ovary	0.06±0.0004	0.06±0.0008	0.06±0.0004	0.06±0.0004

Organ weight (g) of female wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett).

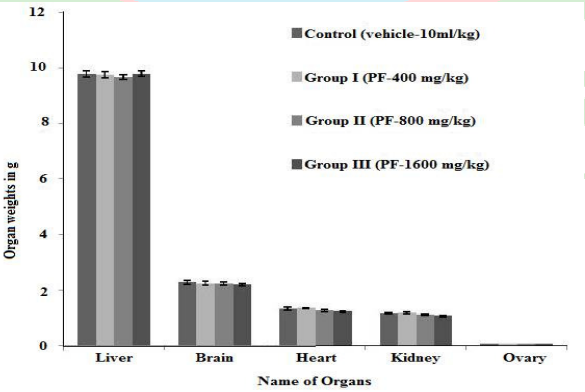


Figure 4: Organ weight of male rats of each groups

Organ weight (g) of female wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett) (Separate graph of Ovary is given below).

PF- Polyherbal Formulation

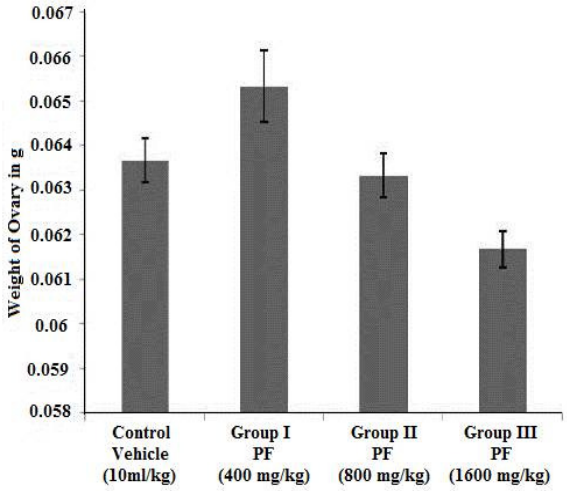


Figure 5: Testis of male rats of each group

Ovary weight (g) of female wistar rats of each group treated during 90 days dosing with vehicle (control) and polyherbal formulation (*p<0.05, **p<0.01: ANOVA - Dunnett).

PF- Polyherbal Formulation

The 90 days treatment groups I,II,III:

There are significant changes observed in relative body weights (Table II, III). No significant changes has observed in relative weights of vital organs (Table IV, V and Figure 1,2,3,4,5) like brain, heart, kidney, liver but there are changes (not significant) in organ weight of ovary, testis and epididymis observed.

In histopathological observation of histological sections of organs like brain, heart, kidney and liver show no any sign of toxicity or lesions or degenerations, while ovary, testis and epididymis show significant changes in histology (Figure 6,7,8).

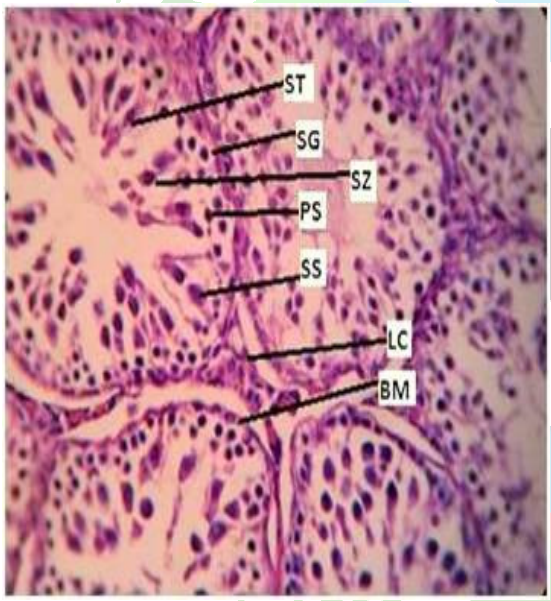
In histological sections of testis (Figure 6) of group III (1600 mg/kg/day) indicating degeneration of testis, seminiferous tubule degeneration, cyst formation, interstitial cell hyperplasia, complete loss of all developing cells indicating atrophy of testes while histological sections of group I (400 mg/kg/day) show increasing in sperm flagella and spermatogenesis than group II (400 mg/kg/day).

Histologial sections of epididymis (Figure 7) of group III (1600 mg/kg/day) indicating azoospermia, degeneration, increase in intertubular space, vacuolation of basement epithelial cell. Epididymis of group I (400 mg/kg/day) has normal histology than group II (800 mg/kg/day) and III (1600 mg/kg/day). Epididymis of group II has contain less mature sperm cells.

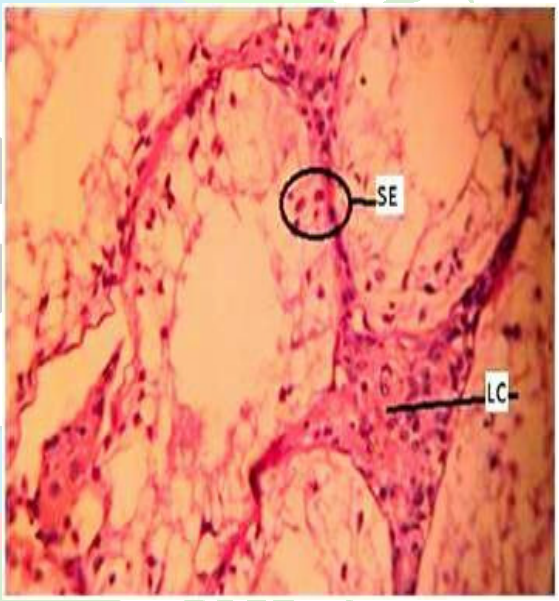
Histological sections of ovary (Figure 8) of group III (1600 mg/kg/day) show atrophy and loss of developing follicles, reduced in size, less number of corpus luteum than group I (400 mg/kg/day) and II (800 mg/kg/day).

Control (distilled water-10 mg/kg)

Group I (400 mg/kg/day)



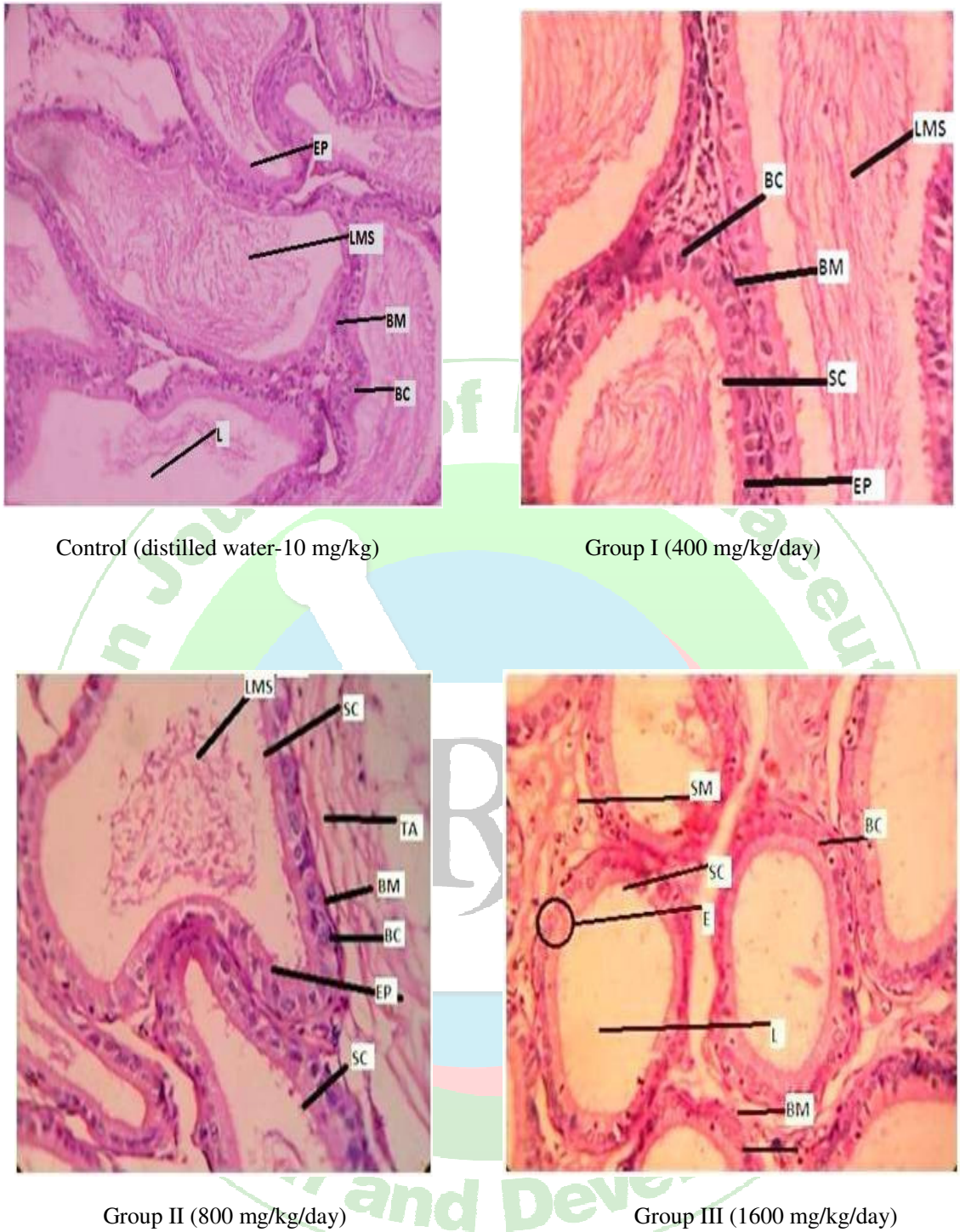
Group II (1600 mg/kg/day)



Group III (1600 mg/kg/day)

Figure 6: Testis of male rats of each group

(control-distilled water, 10 mg/kg/day) shows normal histoarchitecture,
(group I-400 mg/kg/day) shows increase in seminiferous tubule size, maximum no. of sperm flagella,
(group II-800 mg/kg/day) shows spermatogenesis but less than group (I),
(group III-1600 mg/kg/day) Shows degeneration, interstitial cell hyperplasia and atrophy.
ST-Spermatid, LC-Ledig Cell, SZ-Spermatozoa, PS-Primary Spermatocytes, SS-Secondary Spermatocytes, SG- Spermatogonia, SE-Seminiferous Epithelium, ST-Seminiferous Tubules, SF-Sperm Flagella, BM-Basement Membrane, BV- Blood Vessel.



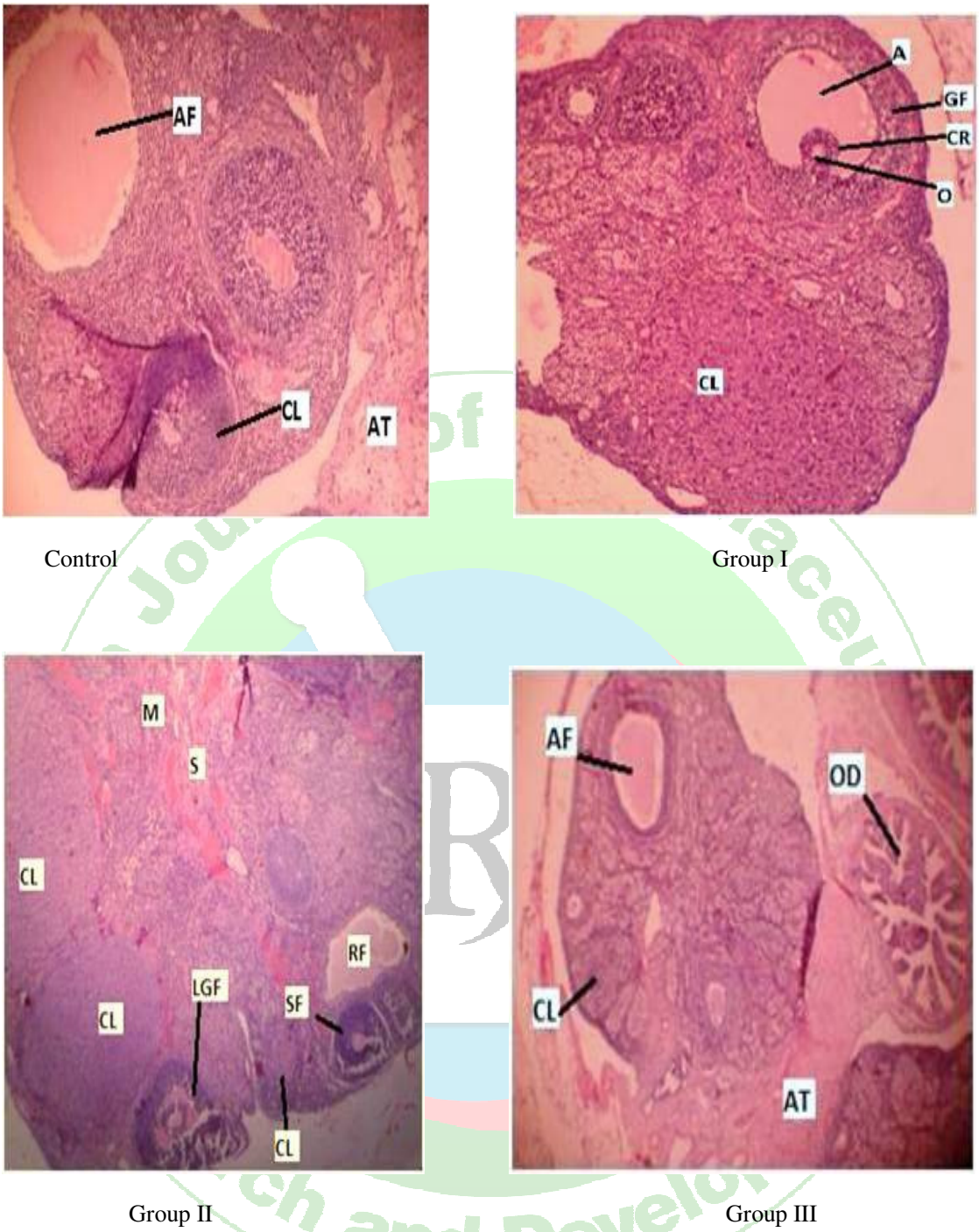
Control (distilled water-10 mg/kg) Group I (400 mg/kg/day)

Group II (800 mg/kg/day) Group III (1600 mg/kg/day)

Figure 7: Epididymis of male rats of each group

(control-distilled water, 10 mg/kg/day) shows normal histoarchitecture,
(group I-400 mg/kg/day) shows presence of mature sperm cells in lumen of epididymis,
(group II-800 mg/kg/day) shows very few mature sperm cells in lumen,
(group III-1600 mg/kg/day) shows azoospermia, degeneration, increase inter tubular space.

E-Epithelium, SC-Stereocilia, BM-Basement Membrane, BC-Basal Cell, EP-Epithelial Cell, L-Lumen, SM-Smooth Muscle, TA-Tunica Albuginea, LMS- Lumen Containing Mature Sperm Cells.



Control Group I

Group II Group III

Figure 8: Ovary of female rats of each group

(control-distilled water, 10 mg/kg/day) shows normal histoarchitecture,
(group I- 400 mg/kg/day) shows normal histoarchitecture, large size of corpus luteum,
(group II-800 mg/kg/day) shows maximum no. of corpus luteum and large size of corpus luteum,
(group III-1600 mg/kg/day) shows atrophy of ovary, less no. of corpus luteum and decreased size of corpus luteum and developing follicles.

A-Antrum, CL-Corpus Luteum, OD-Oviduct, GF-Graafian Follicle, CR-Corona Radiate, PF-Primary Follicle, SF-Secondary Follicle, RF-Ruptured Follicle, O-Oocyte, M-Medulla, S-Stroma, LGF-Large Graafian Follicle, AF-Atretic Follicle, AT-Adipose Tissue.

DISCUSSION

Herbal medicines have been used world-wide for thousands of years. These herbs mainly originate from plants, minerals and animal products and may be used either in their primary, minerals and animal products and may be used either in their primary forms or combined into mixtures. Herbal preparations can also be formulated into tablets, pills and liquids, as well as being commercially available in the form of proprietary medicines. The purpose of medicinal use of herbs may vary with the different traditional medicinal system of different societies, but can simply be considered as being either for the promotion of health or the relief of ailments [22-24].

Traditionally, there is a myth that herbs have been considered to be non-toxic and even harmless, mainly because of their natural origin. A World Health Organization (WHO) survey indicated that about 70-80% of the world's population rely on non-conventional medicine, mainly of herbal source in their primary healthcare [25-27]. Although medicinal plants may produce several biological activities in humans, generally very less data is available about their toxicities [20, 28] and this lack of information makes it difficult to compare the benefit-risk ratio profile of herbal medicines. However, both adverse drug reactions and poisonings associated with the use of herbal medicines have increasingly been reported last few years [20, 24]. In USA, accidental substitution of Planain with *Digitalis lanata* in 1997 used as dietary supplement, led to serious cardiac arrhythmia as side effect and in India, in 1998, mustard oil with *Argemona Mexicana* had caused an epidemic of dropsy [20]. In daily practice, many herbal poisonings were not diagnosed or treated correctly and therefore, more information about herbal toxicology is urgently needed. Though, the medicinal plants are considered to be non-toxic, the sustenance of life, can be toxic, if consumed too much or in an inappropriate proportion. Thus the key aspect to understanding the toxicity of materials is to know how much of a substance dose can cause harm regarding to safety and efficacy. Hence, the scientific approach through experimental and clinical validation of efficacy and documentation of useful herbs, herbal preparations and other formulations is necessary, is done in modern medicine, animal toxicity studies are also required to establish the potential adverse effects [29].

Till today, there is no any acute or sub-acute toxicity found for *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* from various studies [4,11,25,30,31,32]. From many years, all these medicinal plants have been used as an aphrodisiac in inappropriate for maximum time and therefore it is necessary to study chronic toxicity study for safety of this combination. In this chronic toxicity study rats received the polyherbal formulation of *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* for 90 days show there were no treatment related mortality during the study period. The progressive slightly were increased in body weights.

The changes in the organ weights occur due to accumulation of toxicants or necrosis of tissues. However, in the present study, no significant changes were observed in relative organ weight of brain, heart, kidney and liver of control and treated groups. But there are slightly increased in weight of testis, ovary, epididymis and ovary of animals of group I (400 mg/kg/day) as compared to control and other groups and it may be due to increase in spermatogenesis, sperm maturation, increase in sperm count in testis, epididymis and increase in ovulation of ovary by increase in follicle-stimulating hormone (FSH), Luteinizing hormone (LH) [30].

The microscopic analysis of the vital organs of the treated animals like brain, heart, kidney and liver does not show any significant changes in colour, texture, organ swelling and atrophy or hypertrophy as compared to that of control group organs. But there are significant changes in histoarchitecture of organs like ovary, testis and epididymis of group III (1600 mg/kg/day) animals.

In histological sections of testis show seminiferous tubules degeneration, cyst formation, interstitial cell hyperplasia, while group II (800 mg/kg/day) shows increase in

sperm flagella and spermatogenesis but less than group I (400 mg/kg/day). Histological sections of epididymis of male rats of group III (1600 mg/kg/day) show azoospermia, degeneration and increase intertubular space, vacuolation of basement epithelial cell while epididymis of group I (400 mg/kg/day) shows normal histoarchitecture. All these changes in testis and epididymis of group show, there may be increase in FSH, LH and testosterone hormones [30] due to the polyherbal formulation.

In histological sections of ovary of female rats of group I (400 mg/kg/day) and II (800 mg/kg/day) indicate maximum number of primary follicles, secondary follicles and corpus luteum that is the polyherbal formulation may increases estrogen and progesterone hormones. But in group III (1600 mg/kg/day) sections of female rats show loss of developing follicles, reduced size and less number of corpus luteum than group I and II and atrophy of ovary.

Overall, this study provides valuable preliminary data on the toxicity profile of polyherbal formulation of *Tribulus terrestris, Chlorophytum borivilianum* and *Withania somnifera* that should be useful for the planning further studies. The above study needs further mating procedure and measuring of live births along with visible abnormality and post-implantation loss that is the effects of these plants on the fetus in pregnant animals, on the reproductive capacity of animals and on the genetic system and also different steroidal hormonal test.

Conclusion of this study is that polyherbal formulation of *Tribulus terrestris, Withania somnifera* and *Chlorophytum borivilianum* have toxic effects if used in large dose for longer duration of time but this study may also help in estimation of maximum safe dose for longer duration of time. Mutagenicity and carcinogenicity studies are essential to further support the safe use of these plants.

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