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Pragmatic Toxicity Profiling of a Salubrious Polyherbal Combination of Tinospora Cordifolia, Withania Somnifera, and Boerrhavia Diffusa in Swiss Albino Mice

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ABSTRACT

Acute and subacute toxicity screening of novel entrants in the stream of polyherbal formulations opens new doors towards the scientific approach of establishing them in the clinical market. Toxicity profiling asserts the humongous pharmacological potential that the natural herbs hold and establishes their safety profile. To exemplify this statement, this study further deals with acute and subacute toxicity studies of an olive oil-based polyherbal combination (PHC). This combination has been developed with the view that it proves to be clinically effective in diabetic cardiomyopathy and neuropathy. However, until now, the acute and subacute toxicity studies have been done on the developed PHC, and experimentally effective dose combinations have been identified. The follow-up part of this research includes the screening of the PHC against specific in vivo screening models in its initial stages. This research paper strictly summarises the acute and subacute profile of the salubrious polyherbal combination. Three herbs with strong literature background were selected: Tinospora cordifolia, Withania somnifera and Boerrhavia diffusa; and their crude extracts were prepared. The reported ED_{50} values of each of these plants were selected and mixed in 5 different combinations and subjected to Acute Toxicity studies. It was followed by the 28-days subacute toxicity screening of 9 different combinations that were elucidated from the acute toxicity study results. The findings were supported with recovery group studies to conform to any late toxicity symptoms of the polyherbal combination. The findings of this study helped in reaching some pathbreaking conclusions on the polyherbal combination designed.

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INTRODUCTION

Diabetes mellitus, one of the pandemic metabolic disorders, tops the list of pathogenic causes for end-organ failure (Goodman, 2010). The two of the most common macroscopic complications associated with diabetes mellitus are Cardiomyopathy (Haller and Benowitz, 2000) and Neuropathy (Jakkula *et al.*, 2004; Shamim *et al.*, 2018) . The increasing diabetic population in the world and sarcastically decreasing medications that could prevent the pathogenesis of these complications leave us at crossroads of pharmacological management of diabetic complications. Hence, the world of natural

herbs proves a silver lining in the cloud. In this view, a polyherbal combination has been designed in the discussed study with three herbs, namely Tinospora cordifolia, Withania somnifera, and Boerrhavia diffusa (Shamim *et al.*, 2017) .

The present study deals with the acute and 28-days subacute oral toxicity studies of the above mentioned polyherbal combination on Swiss Albino mice as (Palmer *et al.*, 2003; OECD, 2001) . In the acute toxicity study, PHC was administered orally in a single dose to the mice (dose combination levels are discussed later in the paper). Mortality, clinical signs, and change in body weight were monitored for 14 days. Salivation, soiled perineal region, and a decrease in body weight were observed in the extract-treated groups. Mortality was encountered in one of the combinations during the study. Therefore, the approximate LD_{50} of PHC in mice was found to be at the dose level 2^A:2^B:2^C (as per OECD guidelines); A: Tinospora cordifolia; B: Withania somnifera; C: Boerrhavia diffusa. In the subacute toxicity study, PHC was given orally to the mice for 28 days at sub-graduated dose levels between the lethal dose level & the dose level combination 1^A:1^B:2^C. There was no PHC-related toxic effect in the body weight, food consumption, haematology, and clinical chemistry and organ weights except at the dose level (2^A:2^B:2^C). The toxicity induced mortality in this group was further proven by end-organ toxicity signs observed after the necropsy and in the histopathological examination of different organ tissues (Crombie *et al.*, 1992) . Toxic effects in the haematology and serum biochemistry, as well as morphological changes, were observed in the gross organ study. Therefore, it was concluded that all dose level combinations were safe, except for combination 2^A:2^B:2^C (Bisht *et al.*, 2014; Grandhi *et al.*, 1994).

Tinospora cordifolia (Guduchi), Withania somnifera (Ashwagandha), and Boerhaavia diffusa (Punarnava): Herbs of Polyherbal Combination

The plants selected for the polyherbal combination (PHC) are T.cordifolia (Menispermaceae) also commonly known as Guduchi or Giloe, W.somnifera (L) Dunal (Solanaceae), commonly called Ashwagandha, and B.diffusa (Nyctaginaceae), widely known as Punarnava in traditional herbal literature. T.cordifolia is widely used in Indian Ayurvedic medicine for treating diabetes mellitus (Fossati and Prencipe, 1982) . It has been reported in various experimental studies conducted on rodents that the daily administration of both alcoholic and aqueous extract of T.cordifolia potentially controlled the blood glucose level and also enhanced the glucose tolerance (Dwivedi *et al.*, 2012; Patil and Chaudhary, 2013) . Withania somnifera, a plant from the Solanaceae family, also known as Ashwagandha (Sanskrit), is an Ayurvedic Indian medicinal plant, which has been widely used as an antioxidant, antistress, and antihypertensive herb. The phytoconstituents of this plant with their pharmacological and therapeutic potential have been well reported. Different researches state that W.sommifera possess neuroprotective, lipid-lowering, anti-Parkinson's, antineoplastic, and other anabolic activities. It has also been proven fruitful in the treatment of arthritis, geriatric mental issues, and stress. It is one of the most commonly used herbs as an antistress and adaptogenic agent. It is also known to increase life span and has antiageing effects. The roots of W.somnifera contain several alkaloids called withanolides, a few flavonoids, and reducing sugar. The major active compounds of the roots are reported to be withanolides and glycosides (Kuppurajan *et al.*, 1989; Marles and Farnsworth, 1995). Another most typical and exemplary plant of the Ayurvedic plethora is Boerhaavia diffusa, belonging to the Nyctaginaceae family. The whole plant, as well as individual parts like roots, stem, and leaves of B.diffusa, have been extensively used for the treatment of various inflammatory disorders (Aphale *et al.*, 1998; Ujowundu *et al.*, 2008) such as gonorrhoea, dyspepsia, edema, jaundice, dysmenorrhoea, anaemia, liver, gall bladder, and kidney disorders, enlargement of spleen, abdominal pain, abdominal tumours, and cancers. It is also documented as a potent diuretic, laxative, and a menstrual promoter in Indian Pharmacopoeia. It is administered orally as a blood purifier and to relieve muscular pain. Based on these medicinal properties and health benefits, the plants were screened for Polyherbal combination (M and S.S, 2007; Mungantiwar *et al.*, 1997b).

MATERIALS AND METHODS

Collection & Authentication of Plant Material

All the plant materials were purchased from local medicinal plant supplier of Lucknow and authenticated by CSIR-National Botanical Research Institute, Lucknow. NBRI invoice number provided: CSIR/NBRI/003/10.

Preparation of Plant Extracts

The roots of Tinospora cordifolia (500 g) were collected and shade dried to form a powdered mixture. It was subjected to sohxelation process with double distilled water and ethanol combined in the ratio of 1:1 for 36 hours at 40°C. The liquid extract was cooled and dried by evaporating its contents on

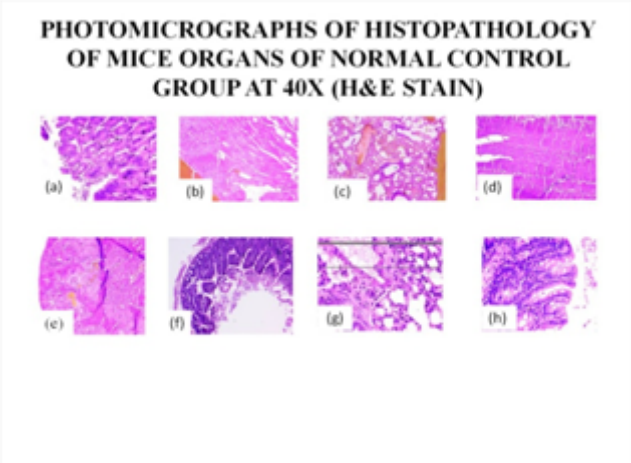

Figure 1: (a) Stomach; (b) Heart; (c) Lungs; (d) Brain; (e) Intestine; (f) Kidney; (g) Liver; (h) Pancreas

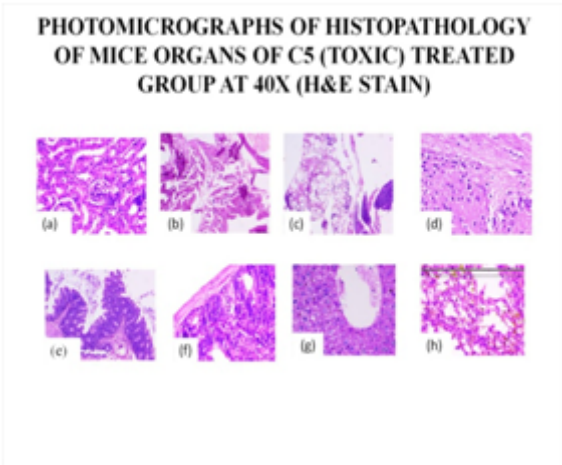

Figure 2: (a) Stomach; (b) Heart; (c) Lungs; (d) Brain; (e) Intestine; (f) Kidney; (g) Liver; (h) Pancreas

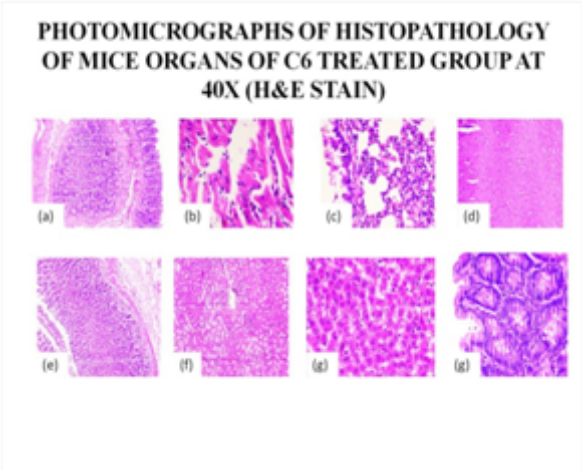

Figure 3: (a) Stomach; (b) Heart; (c) Lungs; (d) Brain; (e) Intestine; (f) Kidney; (g) Liver; (h) Pancreas

Table 1: Ratios of plant extracts in polyherbal combination for Aute toxicity studies

S.no.	Combination	Avg Animal weight (g; n=3)	T.cordifolia	W.somnifera	B. diffusa
1	C1	20.73	1:	1:	1
2	C2	22.55	2:	1:	1
3	C3	21.65	1:	2:	1
4	C4	22.55	1:	1:	2
5	C5	24.66	2:	2:	2

(Note: Theratios were designed based OECD guidelines for oral acute toxicity studies.)

Table 2: Developed ratios of PHC elucidated from findings of Acute toxicity studies

S.no.	*Dose level Combination	Avg Animal weight (g; n=3)	*T.cordifolia*	*W.somnifera*	*B. diffusa*
1.	Normal control	21.55	NIL (20% Glycerol)		
2.	C5	22.73	2:	2:	2
3.	C6	22.65	1.3:	1:	2
4.	C7	21.29	1.6:	1:	2
5.	C8	23.45	1.9:	1.3:	2
6.	C9	19.86	1:	1.3:	2
7.	C10	20.78	1:	1.6:	2
8.	C11	21.02	1:	1.9:	2

(*Note:The doselevels were selected based on the result of a preliminary 2-week acute toxicity study.)

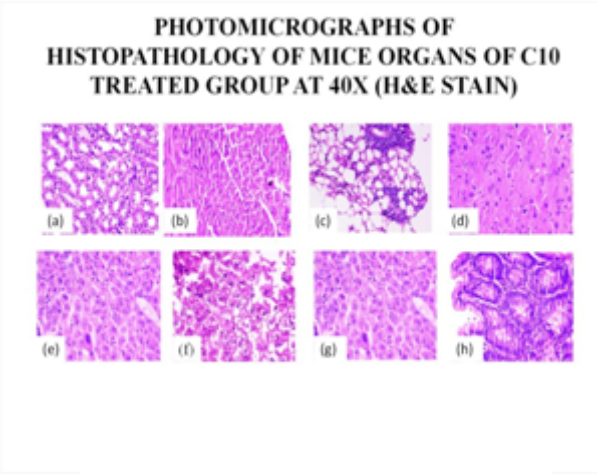

Figure 4: (a) Stomach; (b) Heart; (c) Lungs; (d) Brain; (e) Intestine; (f) Kidney; (g) Liver; (h) Pancreas

rota vaporiser. The yield of hydroalcoholic extract was 17.55% w/w of crude drug power. The extract was stored at a temperature of approximately 1–1.5 °C (Mungantiwar *et al.*, 1999) .

The roots of Withania somnifera were used to prepare the extract. The roots were pulverised using a grinder and then extracted with methanol. The extract was then concentrated on a rotator evaporator to distil methanol and get a thick paste. 250 g of the thick paste of methanolic extract was again extracted with 500 ml of ethyl acetate in Soxhlet extractor for 3 hours at a temperature of 65 °C and then concentrated on the rotator evaporator (Dhundi *et al.*, 2011).

The collected plant materials of Boerrhavia diffusa were shade dried and powdered mechanically. 200g of pulverised material was soaked in 100 ml of chloroform and methanol each for 48 hours, following the principle of maceration. It was then filtered. The solvent was distilled out from the filtrate and dried under reduced pressure in Rota vapour (Sharma *et al.*, 1985).

Table 3: Detailed Effects of toxicity induced by PHC in mice during 14 day acute toxicity study

S.No	Parameters	Groups					
		Normal control	C1	C2	C3	C4	C5
General appearance							
1.	Avg. Initial Body weight (g)	21.02	20.73	20.47	21.30	19.86	20.55
2.	Avg. Final body weight (g)	22.55	21.55	20.80	21.85	20.65	21.88
3.	Stool Color	Black	Black	Black	Black	Black	Black
4.	Diarrhoea	NIL	NIL	NIL	NIL	NIL	NIL
5.	Mucoid Stool	NIL	NIL	NIL	NIL	NIL	Sticky stool
6.	Food consumption in 24 hr (g)	35.00	34.00	36.00	33.00	32.00	25.00
7.	Water consumption in 24 hr (ml)	18	20	22	26	23	30
8.	Visible abnormalities	NIL	NIL	NIL	NIL	NIL	NIL
9.	Rate of respiration	Normal	Normal	Normal	Normal	Normal	Increased
10.	Drowsiness	Not observed	Not observed	Not observed	Not observed	Not observed	Observed
11.	Lethargy	Not observed	Not observed	Not observed	Not observed	Not observed	Observed
12.	Rashes	Not observed	Not observed	Not observed	Not observed	Not observed	Not Observed
Hypersensitivity reactions							
13.	Skin color	Normal	Normal	Normal	Normal	Normal	Normal
14.	Eye color/pigmentation	Normal	Normal	Normal	Normal	Normal	Normal
Behavioral parameters							
15.	Paw licking	Not Observed	Observed	Observed	Observed	Observed	Observed
16.	Paw Jumping	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
17.	Paw Biting	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed
18.	Mobility (No. of movement in 15 min)	33	32	34	34	35	27
19.	Mortality	Alive	Alive	Alive	Alive	Alive	Dead

Preparation of the Polyherbal Combination

Once the extract for all the three test materials was prepared, it was dried and suspended in olive oil mixed in the following combinations; 20% glycerol was used as a suspending agent. The different dose levels of the drugs were mixed in different ratios discussed later under experimental design.

Acute Toxicity & Subacute Toxicity Study

The present study was conducted at the Departmental Animal House, Faculty of Pharmacy, Integral University as per Good Laboratory Practices and following the protocol of the Organization for Economic Cooperation and Development (OECD, 2001) .

The study protocols were also reviewed and approved by Institutional Animal Ethical Committee (IAEC), Faculty of Pharmacy, Integral University, and Lucknow. Reg no. 1213/PO/Re/S/08/CPCSEA, 16^{th} June 2017; Approval no. IU/IAEC/ 17/04.

Treatment with Polyherbal Extract

The Polyherbal Combination (PHC) was administered using an oral gavage needle. In acute toxicity study, prior to dosing, animals fasted overnight

Table 4: Hematological Studies

Parameters	Normal control	C5	C6	C7	C8	C9	C10	C11	Recovery Control	Recovery Group
RBC Count (106/ μL)	7.52± 0.061	3.85± 0.045*	7.55± 0.05	7.47± 0.087	7.52± 0.023	7.5± 0.035	7.5± 0.026	7.5± 0.015	7.50± 0.02#	7.49± 0.03#
WBC Count (103/ μL)	8.77± 0.026	2.03± 0.183*	8.12± 0.098	8.19± 0.393	8.29± 0.373	8.24± 0.32	8.74± 0.015	8.70± 0.021	8.71± 0.021#	8.69± 0.02#
Platelet Count (103/ μL)	1122.33± 2.517	491.33± 23.24*	1119.33± 4.16	1113.33± 13.31	1120± 2.646	1118.667± 2.51	1085.33± 37.84	1123± 1.00	1117.67± 2.51#	1119.33± 2.51#
DLC: Eosinophils (%)	1.1± 0.1	0.09± 0.01*	0.8± 0.1	0.833± 0.208	0.933± 0.153	0.9± 0.2	1.067± 0.15	1.133± 0.115	1.18± 0.02#	1.173± 0.02#
DLC: Neutrophils (%)	17.433± 0.153	4.633± 0.757*	17.043± 0.232	16.85± 0.573	17.093± 0.11	16.740± 0.444	17.4± 0.2	17.367± 0.153	17.127± 0.127#	17.06± 0.08#
Haematocrit (%)	44.057± 0.031	19.72± 2.119*	44.033± 0.015	44.237± 0.228	43.933± 0.775	43.943± 0.061	43.94± 0.12	43.703± 0.575	43.93± 0.053#	44.007± 0.025#
Haemoglobin (g/dL)	11.837± 0.272	3.96± 0.147*	11.947± 0.169	11.94± 0.164	11.973± 0.101	11.943± 0.119	12.07± 0.02	12.073± 0.006	11.943± 0.05#	11.97± 0.046#
Blood Glucose level (mg/dl)	133.667± 2.082	233.00± 2.646*	145.00± 3.00	147.33± 3.055	154.33± 2.517	155.00± 6.928	134.667± 2.517	136.667± 2.082	132.667± 1.528#	134.333± 1.528#

All values are expressed as Mean ±SD (n=3). The values were found to be significantly different at *p<0.05 when compared with normal control. The values of recovery group were compared with recovery control and the difference found to be non significant at #p<0.05

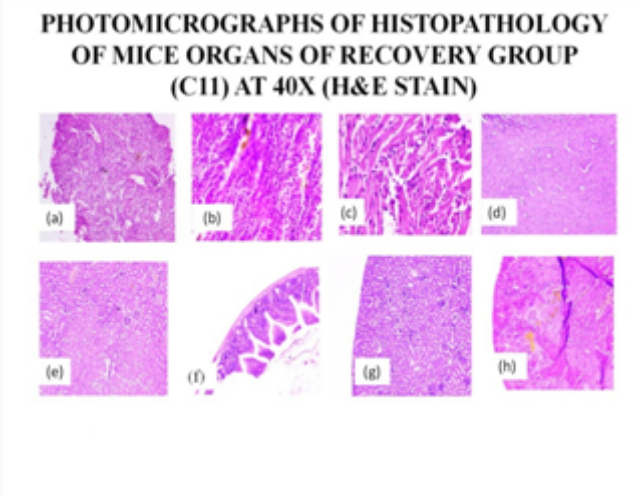

Figure 5: (a) Stomach; (b) Heart; (c) Lungs; (d) Brain; (e) Intestine; (f) Kidney; (g) Liver; (h) Pancreas

Table 5: Serum Biochemistry

Parameters	Normal control	C5	C6	C7	C8	C9	C10	C11	Recovery Control	Recovery Group
Total Cholesterol	75± 2.646	177.00± 4.359*	74.667± 1.528	71.00± 2.00	71.667± 2.082	74.667± 4.163	73.667± 3.215	72.00± 1.00	71.667± 0.577#	76.00± 1.0#
Triglycerides	34.00± 2	134.00± 10.149*	33.00± 2.646	33.667± 3.055	32.00± 1.732	35.00± 3.00	37.33± 2.517	34.667± 3.055	33.00± 1.00#	30.33± 0.577#
SGOT (mg/dL)	27.079± 2.65	248.00± 2.00*	28.00± 2.646	28.00± 2.00	25.33± 3.125	27.33± 3.055	25.33± 4.163	25.667± 3.125	27.33± 2.082#	25.667± 2.082#
SGPT (mg/dL)	26.00± 2.00	304.33± 2.52*	26.00± 1.00	24.00± 2.00	26.00± 2.00	27.33± 2.517	29.667± 4.041	25.667± 4.041	20.667± 2.082	20.33± 2.082#
SAP(mg/dL)	156.67± 7.64	421.00± 3.61*	151.67± 19.088	166.33± 7.767	225.00± 25.00	175.33± 17.214	171.33± 7.095	168.67± 6.506	162.00± 2.00#	164.33± 3.055#
Total serum Bilirubin (mg/dL)	0.4± 0.1	2.37± 0.042*	0.3± 0.1	0.4± 0.1	0.5± 0.1	0.7± 0.1	0.7± 0.1	0.8± 0.1	0.82± 0.03#	0.823± 0.021#
Direct Serum Biirubin(mg/dL)	0.19± 0.01	1.19± 0.01*	0.167± 0.015	0.233± 0.015	0.163± 0.025	0.207± 0.015	0.173± 0.015	0.203± 0.021	0.217± 0.015#	0.22± 0.03#
Indirect Serum Bilirubin(mg/dL)	0.397± 0.045	1.12± 0.01*	0.46± 0.031	0.38± 0.046	0.44± 0.021	0.33± 0.046	0.35± 0.05	0.53± 0.03	1.217± 0.012#	1.22± 0.03#
Serum Urea(mg/dL)	24.24± 4.24	6.33± 1.528*	21.96± 7.57	26.12± 7.54	19.04± 5.63	32.40± 1.005	23.81± 1.065	27.69± 3.175	23.52± 0.282#	23.85± 0.187#
Serum Creatinine(mg/dL)	0.62± 0.047	0.17± 0.01*	0.58± 0.05	0.61± 0.059	0.697± 0.025	0.56± 0.026	0.6± 0.05	0.57± 0.044	0.587± 0.035#	0.583± 0.015#
Serum Potassium(mg/dL)	4.13± 0.321	0.573± 0.087*	3.967± 0.208	4.397± 0.332	3.923± 0.892	4.397± 0.52	4.06± 0.036	3.797± 0.168	3.677± 0.05#	3.683± 0.02#
Blood Urea Nitrogen	12.00± 2.00	4.67± 2.082*	13.00± 2.00	14.00± 2.00	9.67± 2.082	11.33± 1.528	14.67± 2.517	12.33± 1.528	12.8± 0.31#	12.74± 0.571#

Allvalues are expressed as Mean ±SD (n=3). The values were found to besignificantly different at *p<0.05 when compared with normal control. Thevalues of recovery group were compared with recovery control and the differencefound to be non significant at #p<0.05

Table 6: Relative Organ Weights

Organ	Normal control	C5	C6	C7	C8	C9	C10	C11	#Recovery Control	#Recovery Group
Average body weight (g)	19.47	21.91	20.94	22.2	21.19	20.5	22.51	22.4	21.56	20.72
Brain	10.505±0.049	5.567±0.823*	9.884±0.699	9.783±0.854	10.751±1.813	10.287±0.942	10.868±1.154	10.361±1.259	9.656±1.041	9.783±0.854
Heart	2.876±0.051	4.959±0.65*	2.577±0.356	2.163±0.328	2.56±0.121	2.637±0.16	2.366±0.409	1.994±0.433	2.436±0.35	2.163±0.328
Lungs	6.009±0.05	9.217±0.33	5.476±0.2	5.374±0.075	5.071±0.406	5.629±0.204	4.93±0.226	4.939±0.171	5.424±0.423	5.374±0.075
Stomach	14.432±0.044	7.7±2.06*	11.812±2.174	12.158±0.563	13.272±1.412	13.685±0.209	12.727±0.727	12.274±0.371	12.494±0.948	12.158±0.563
Liver	37.819±0.138	42.074±2.582*	34.078±1.556	32.503±1.001	34.066±1.98	36.813±1.212	31.355±0.553	32.752±1.066	34.197±2.946	32.503±1.001
Intestine	62.064±0.453	47.802±1.178*	66.355±3.843	61.729±2.19	64.571±7.046	66.148±3.526	61.281±5.851	60.175±2.363	63.865±5.592	61.729±2.19
Pancreas	11.111±1.284	23.691±2.178*	9.664±0.236	9.187±0.167	9.515±0.479	9.974±0.408	9.018±0.199	9.242±0.36	9.809±0.547	9.187±0.167
Kidneys	7.511±0.644	1.851±2.344*	8.806±0.454	8.436±0.134	8.679±0.399	8.394±0.951	8.101±0.71	7.511±0.644	8.094±0.52	8.436±0.134
no. of animals	3	0	3	3	3	3	3	3	3	3

All values are expressed as Mean±SD (n=3). *The values were found to be significantly different at *p<0.05when compared with normal control. # The values of recovery groupwere compared with recovery control and results were found to be nonsignificant at *p<0.05

for approximately 16 hrs. Drinking water and food were provided ad libitum. The dosing was done once a day for 14 days without fasting (Unuofin *et al.*, 2018). Similarly, for subacute toxicity studies, the dosing was continued for 28 days with dose levels screened from the outcomes of acute toxicity studies. Two groups were dosed for an extended two week recovery period. The extract dosing volume was kept as 0.5mL/kg/day, and the dose was determined based on measured body weights of animals (as it varied throughout the study) for both acute and subacute toxicity studies (OECD, 2001).

Experimental Animals

The Swiss albino mice (4-week old) (18-22gm) used in the acute toxicity studies were retrieved from animal husbandry of Central Drug Research Institute (CDRI), Lucknow. During the study period, the animals were housed at a temperature of 22 ± 3 °C and relative humidity of 50 ± 20%, with 12 hr light and dark cycle (Mungantiwar *et al.*, 1997a). The animals were acclimatized to laboratory conditions for 7 days before beginning activity. Groups of three animals were housed in polypropylene cages (260 ×350 × 210 mm) during the acclimatization and one animal per cage during the dosing periods. Proper marking and identification codes were assigned to each cage to distinguish from oth-

ers. Animals were given standard pellet diet and drinking water ad libitum throughout the study period (Vishal *et al.*, 2009).

Experimental Design

Female Swiss mice (4-week old) (18-22gm) were divided into 6 groups of 3 animals each. The 1st group served as a Normal control and received 20% glycerol. At the same time, 2nd, 3rd, 4th, 5th, and 6th were considered as toxic groups received orally different dose ratios of polyherbal combination (dissolved in 20% glycerol) for individual herbs (Lee *et al.*, 2012; Evivie, 2016). The dose combinations given were as follows

All animals were observed for clinical symptoms like convulsion, tremor, constipation or diarrhoea, and changes in eye and skin colours, if observed. Behavioral activities such as changes in body weight, food intake and water intake, and rate of respiration were also closely monitored at the interval of 30, 60, 120, 240, and 360 min after the first dose and twice a day after that for the 14-day experimental activity (Unuofin *et al.*, 2018; Han *et al.*, 2015). The body weights were recorded before dosing as well as on days 1, 3, 7, and 14 after dosing (Ojo and Mahre, 2010). The detailed results have been illustrated in Table 3.

The results of the acute toxicity studies showed that all the combinations of the three plant extracts were safe except the C5 combination (2^A:2^B:2^C); (Table 3). Upon administration of the plant extracts in the C5 ratio, certain physiological symptoms of toxicity such as sticky stool, increased rate of respiration, drowsiness, lethargy, increased paw licking, reduced food consumption, and mortality were observed. However, subacute toxicity studies were conducted to confirm that the death resulted from combination induced toxicity and to countercheck the other combinations as well.

The dose levels were screened from the outcomes of acute toxicity study. The low dose level was set at the combination of 1^A:1^B:2^C at which no mortality was observed, and the high dose level was set at the combination of 2^A:2^B:2^C, at which mortality was observed in acute toxicity study. The doses in between were set at different ratio combinations (increasing by 1/3 rd ratio of initial dose) ranging between the two selected dose levels from Table 1 . Control animals were administered with vehicle only (20% glycerol). 24 animals of either sex mice (6-week-old) per group were administered PHC at dose combinations mentioned in Table 1. Additionally, three animals each were added to the control and high dose groups for the recovery study. The purpose of repeating the C5 combination subacute toxicity studies was to assure that mortality observed was due to dose-related toxicity and not due to any other reason like choking, lack of drinking water etc. The following Table 2 illustrates the graded dose level combinations selected for subacute toxicity studies.

Test Parameters

The animals were thoroughly observed once in a day for clinical signs of any toxicity and twice a day for mortality for the entire study period. The animals in the graded dose groups were observed for two weeks, and those of the recovery groups were observed for extended two weeks without any dosing during the extended period to study any late signs of toxicity. Before dosing on day 1, body weights were recorded. Similarly, body weights were measured once in a week and during the dosing and recovery period and analysed statistically, excluding body weights measured on the day of necropsy to avoid discrepancy in data, since all animals were fasted for 24hrs before sacrificing. The food consumption was measured daily from the day of group assignment to the last day of dosing. During the dosing and recovery periods of subacute toxicity study, the average of daily food consumption was calculated from the total amount consumed in 7 days.

On the 28^{th} day of study, necropsy was performed on all animals (except the recovery group), but only after ensuring that all animals were fasted overnight for approximately 18 hrs. The animals were anaesthetized with thiopental sodium, and blood was collected through retro-orbital plexus and tail vein in two tubes: one with EDTA for immediate analysis of haematological parameters and biochemical estimations, the other without any additives was centrifuged at 4,000 rpm at a temperature of 4 °C for 10 min. Plasma and serum were stored at -18 °C until analyzed for biochemical parameters. The following haematological parameters were evaluated: Erythrocyte (RBC) count, haemoglobin (Hb), hematocrit (HCT), thrombocyte count, leucocyte (WBC) count, differential leucocytes (DLC) count, and Random blood glucose level (RBG) (Singh and Singh, 2017; Han *et al.*, 2015). The remaining blood samples collected for the haematological examination was centrifuged at 3,000 rpm for 10 min to obtain the serum (Papageorgiou *et al.*, 1999; Pearlman and Lee, 1974). The following parameters were then analyzed in serum: Alanine Transaminase/SGPT, Aspartate transaminase/SGOT, Serum Alkaline Phosphatase (SAP) (Pennock *et al.*, 1973),

total bilirubin (Kanezawa *et al.*, 1984; Olson *et al.*, 1990), blood glucose, total cholesterol, triglyceride, total protein, albumin, blood urea nitrogen, and creatinine (Slot, 1965). The electrolytes concentration, including Na+, K+ and Cl− ions, were also estimated (Gokarn *et al.*, 2017). All the results of haematological as well as serum biochemistry assessments are given in Tables 4, 5 and 6.

On the 28^{th} day, after collecting blood samples, the animals were weighed, anaesthetised with thiopental sodium, and sacrificed after the brain, lungs, heart, stomach, intestine liver, spleen, and kidney were dissected out washed with ice-cold saline and weighed. Complete cross-examinations of the outer and inner surfaces of vital visceral organs were performed on all animals on the day of necropsy, and all grossly visible signs of toxicity were recorded. The following organs were weighed (weight of the paired organs was recorded together): brain, heart, lungs, pancreas, liver, stomach, intestine, and kidneys. The organ weights were expressed as relative organ weights with respect to animal body weight. (Roeschlau *et al.*, 1974; Wilkinson *et al.*, 1969) .

The organs were then blotted with filter paper and mounted in organ container bottles filled with 10% formalin for histopathological examinations. 10% tissue homogenates of liver were prepared by homogenising a weighed amount of tissues in 0.1M trisaminomethane-HCl buffer (pH: 7.4). The homogenates were then sent for histopathological estimations to Alpine Diagnostics, Kapoorthala, Lucknow. The investigations on all the tissues of isolated organs from the animals of the normal control and treated groups were carried out post-treatment with paraffin, and staining with hematoxylin and eosin (H&E) stain for microscopic evaluation at 40x resolution (Rifai *et al.*, 2000; Pittler and Ernst, 2003). The images of histopathology are shown in Figures 1, 2, 3, 4 and 5.

Statistical Analysis

The statistical analyses were performed using the statistical analysis software graph pad prism 6.0 for data, including body weight change, food consumption ratio, haematological parameters, serum biochemistry, and relative organ weight. The results were reported as means ± standard deviation. Non-parametric paired t-test was performed to test the homogeneity of variance (*p<0.05); Student t-test was conducted (two-tailed) on each data sets of the individual quantitative parameter.

RESULTS AND DISCUSSION

During the acute toxicity study period, one male rat died on day 2 in the (2:2:2; Toxic group) C5-treated group. The other dose level groups showed no toxicological signs or symptoms. Therefore, we concluded that all the dose level treated groups except C5-treated group had no toxicological outcomes. The food consumption results reflected that mice treated with all the combinations at weeks 2 and 3, consumed lower amounts of feed than the control group, temporarily, which improved later on. However, in the case of C5-treated group where the food consumption by animals was considerably reduced before mortality. The change in body weight pattern in both study groups and recovery groups were comparable to normal control with no significant difference from the normal control, except in the case of C5-treated group where a marked decrease in body weight was observed, and the results were significantly different from normal control at *p<0.05. The graphical analysis of the relative organ weight justifies the toxicity observed in C5-treated group. There was a marked reduction observed in the organ weights of brain, stomach, intestine, and kidneys due to necrosis when compared to their respective normal control. Similarly, there was toxicity related hypertrophy and increased organ weight observed in the heart, lungs, liver, and pancreas (found to be significantly different at *p<0.05 with normal control). The relative organ weights of the other combination-treated groups and recovery groups were comparable to their respective control groups and significantly not different from the normal control. The results of biochemical estimations of various enzymatic parameters in all the ratios of PHC groups and recovery groups showed results within a normal range compared to the normal control and significantly not different from the normal control group except for C5-treated group that showed prominent signs of toxicity in various enzymatic levels. In the liver function test profile, it was observed that serum levels of SGOT, SGPT, total bilirubin, and direct and indirect serum bilirubin were found to be significantly different at *p<0.05. All the LFT parameters were found to be elevated when compared to normal control. Similarly, the kidney function test profile showed reduced levels of serum creatinine, serum urea, and blood urea nitrogen when compared with normal control at *p<0.05 in the C5-treated group. The pattern of other parameters such as AST, ALT, blood glucose, total cholesterol, and triglycerides was found to be elevated significantly than normal control at *p<0.05 in C5-treated group. The revelations from the haemato-

logical profile of all the combinations-treated group indicated that only the C5-treated group had toxic effects on blood-related parameters that differed significantly from normal control at *p<0.05 and all the other combinations showed results comparable to normal control. As evident in the Differential Leukocyte Count Profile, all the cellular values (Monocytes, Lymphocytes, Basophils, Neutrophils, and Eosinophils) were found to be reduced when compared to normal control. The recovery group showed results comparable to its control group with no significant difference. The histopathological examination of various organs from the normal control group, C5-treated group, and recovery group showed normal architecture, suggesting no detrimental changes and morphological disturbances caused by the administration of polyherbal extract in the above combinations, except for the C5-treated group. In the C5-treated group, the following toxic signs were observed (Greaves, 2011). The results of the end-organ study and gross morphology revealed discoloration in the lung, specifically observed in the left lobe and right caudal lobe. In addition, inflammation of the left lobe and congestion of the right caudal lobe were discovered in the histopathological examination. The heart was reported to show early myocardial ischaemia, necrosis with loss of cross striations, oedema, haemorrhage, and early neutrophilic infiltrate. The kidneys showed coagulative necrosis specifically congestion around collecting tubules; the histopathological reports stated tubular necrosis. The H&E stained section of intestine showed marked villous necrosis. The epithelial lining of villi was completely shed off, and there was marked oedema with fibrinopurulent exudates that extended up to muscularis mucosae, acute ischaemic colitis was concluded. The H&E stained section of the stomach showed mucosal coagulative necrosis with superficial erosion and fibrinopurulent exudates there was also mild transmural infiltration by acute inflammatory cells, coagulative necrosis, and gastritis concluded. The liver showed centrilobular necrosis. The H&E stained section showed inflammation with greyish white exudates. The H&E stained section showed focal centrolobular necrosis. There was a cluster of hepatocytes showing coagulative necrosis along with mild but acute infiltration, centrolobular necrosis of liver concluded. The H&E stained section showed mild acute inflammation and spongiosis.

CONCLUSIONS

The acute and subacute toxicity studies revealed interesting and noteworthy facts about the safety profile of the polyherbal combination constituted from the extracts of T.cordifolia, W.somnifera, and B.diffusa. It was concluded from this study that different dose combination ratios of these medicinal plants are safe at most dose levels except when combined at twice ED_{50} of each plant, i.e. at C5 combination, that showed prominent toxicity in the animals as proven in serum biochemistry, haematology, relative organ weights, change in body weights, and histopathological findings. The results of parameters in all other dose combinations were comparable to the normal control at *p<0.05. The use of C11 combination was repeated in the recovery group as it was the highest level of safe dose combination. All the profiles, LFT, KFT, serum biochemistry, DLC, relative organ weights, and change in body weight were comparable to the recovery control with no significant difference in the data at *p<0.05. Hence, the acute and subacute toxicity screening advocates that the given polyherbal combination of the extracts of T.cordifolia, W.somnifera, and B.diffusa based in olive oil is a highly salubrious combination and it can be investigated further for its antidiabetic, cardioprotective, and neuroprotective potentials. The LD50 reported for each of these plants in different literatures reflect that T.cordifolia and B.diffusa (LD50>2000 mg/Kg) are highly safe. The plant that remains in question is W.somnifera. The toxicity observed in C5 combination of PHC may be attributed to the low LD50 of W.somnifera (LD50 = 1750 mg/Kg) and interaction between different phytoconstituents at high dose levels (2^A:2^B:2^C). This study serves as a stepping stone to develop a novel formulation in future to cope with the two most common complications of diabetes: cardiomyopathy and neuropathy.

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Conflict Of Interest

The authors declare that they have no conflict of interest.

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