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Assessment of acute, sub-acute, chronic and genotoxicity of polyherbal formulation DCD-684 in mice

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Abstract: Digas colic drops (DCD-684) a polyherbal formulation containing *Carum carvi, Foeniculum vulgare, Mentha arvensis, Mentha piperita* and *Zingiber officinale* is widely used in Pakistan against gastrointestinal ailments including infantile colic. The DCD-684 (0.03-3ml/kg.bw) administered orally in acute (7-days) and sub-acute toxicity (14-days) tests, displayed neither mortality nor toxicological changes in physical, behavioral, biochemical and histopathological parameters. In chronic study (90-days), DCD-684 (0.3-12 ml/kg.bw) also revealed no changes. However, at 18 and 36 ml/kg.bw, liver demonstrated mild inflammation correlating with raised aspartate transaminase (AST), alkaline phosphatase (ALP) and alpha fetoprotein (AFP) levels. Increased levels of urea and inflamed renal parenchyma indicated mild nephro-toxicity with high alanine aminotransferase (ALT) at 36 ml/kg.bw. The LD_{50} of DCD-684 in mice was 27.5 ml/kg.bw. In hepatocytes at 36 ml/kg.bw, elevated mRNA expression of pro-inflammatory chemokines and cytokines were evident. DCD-684 neither damaged DNA nor induced cytotoxicity in micronucleus assay. In conclusion, polyherbal DCD-684 caused neither hepatic, renal, genotoxicity nor any undesirable effect in mice. Higher doses administered for 90 days showed mild toxic effects with no sign of necrosis, fibrosis or genotoxicity. Thus, in mice DCD-684 demonstrated a wide margin of safety to be used for the relief of infantile colic.

Keywords: Digas colic drops, polyherbal formulation, chronic toxicity, genotoxicity, micronucleus assay, pro-inflammatory mRNA expression.

INTRODUCTION

Plant-derived formulations or medicines have attracted enormous attention worldwide due to the undesirable side effects and other safety concerns associated with synthetic medicines. Therefore, in the developed countries, most of the cases in the primary health management systems seem to be preferring to a combination of several phytochemicals and polyherbal formulations with multiple pharmacological targets treating various diseases (Li *et al.*, 2017). However, there is a slight misconception about them for being naturally safe for health care without any adverse and significant side effects (Markman, 2002; Laukova *et al.*, 2018). Possibly, irrational formulation and processing of the herbal medicines and undesirable interactions with other medicines may lead to unfavorable responses (Fatima and Nayeem, 2016). In the current era of evidence-based medicine, detailed evaluation of the toxicity of polyherbal formulation (s) and their components along with clinical studies is crucial (Shan *et al.*, 2020).

The toxicological complexities accompanied with the use of polyherbal medicines include i.e. liver toxicity or malfunction, hematological and renal toxicity leading towards serious adversarial fatalities like cardiovascular, neurological and psychiatric disorders. Hence, their toxicological and pharmacological properties should be evaluated preclinically for new drug development to warrant the outstanding quality and safety for patients use (Fu *et al.*, 2019; de Brito, 2012).

An essential part of the drug development process includes evaluation of the dosage-, specie- and organ-specific toxic outcomes of an investigational drug (Parasuraman, 2011). Therefore, in various emerging countries employed with the vigorous manufacturing and consumption of herbal medicinal products (HMP) have regulatory bodies which emphasize on the evidences that the product is non-toxic to consume under the prescribed specifications. These regulations must be in alliance with WHO International Regulatory Cooperation for Herbal Medicines (IRCH), instituted in 2005 to promote and protect public health and safety measures globally (Neergheen-Bhujun, 2013).

The polyherbal natural formulation DCD-684 is best known for its remedial use in the management of intestinal colic pain and various gastric disturbances in infants. It is manufactured and marketed by Medics Laboratories Pvt. Ltd., Karachi, Pakistan. This

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formulation contains decoctions of five different plants including *Mentha piperita L*. (peppermint), *Mentha arvensis L*. (corn mint), *Carum carvi* (zeera), *Foeniculum vulgare* Mill (fennel) and *Zingiber officinale Roscoe* (ginger). Each of the individual herbal ingredients of DCD-684 are documented in Unani, Ayurvedic and other traditional systems of medicines for multiple pharmacological properties including anti-spasmodic, carminative and digestive agent which contributes in the management of abdominal colic pains in children. All of these plant components are included in the generally recognized as safe (GRAS) list of Code of Federal Regulations as "spices and other natural seasonings and flavorings" (FDA, 2020). They have also been individually reported in scientific literature to be safe and also accompanied by immuno-protective effects for both animal and human consumption. The extracts of *M. piperita, M. arvensis, F. vulgare and C. carvi* have protective effects against CCl_4 induced hepatic toxicity and fibrosis in rat (Bellassoued *et al.*, 2018; Patil and Mall, 2012; Liu *et al.*, 2009; Samojlik *et al.*, 2010) while *M. piperita, F. vulgare* and *C. carvi* also possess nephro-protective effects in rats and rabbits (Alsalame *et al.*, 2018; Sadrefozalayi and Farokhi, 2014; Abou El-Soud *et al.*, 2014). The extract of *Z. officinale* has improved liver and renal function by suppressing the gene expression of hepatic inflammation markers TNF-*α* and IL-6 and also reduced Fe-induced toxicity in rats (Gholampour *et al.*, 2017; Li *et al.*, 2012). Despite of being popularly claimed to be naturally safe, there is no published information available regarding safety of their combined formulation. Hence, this study was intended to evaluate the safety profile and possible toxicological effects for long-term use of the polyherbal formulation DCD-684 in mice.

MATERIALS AND METHODS

DCD-684 preparation

DCD-684 used in the subsequent experiments were provided by the phytochemistry section, Medics Laboratories (Pvt.) Ltd. These samples were prepared according to the processes mentioned in the WHO Guidelines regarding Good Manufacturing Practices (GMP) for Herbal Medicines (Organization, 2007).

Animals and ethics

In the study, NMRI mice weighing (22-28g) of both genders were used. These were acquired and kept in the Laboratory Animal Science (LAS), Dow University of Health Sciences (DUHS) Karachi, Pakistan. The animals were housed at 22±2°C with controlled humidity (50±10%), 12h light and dark cycle and fed standard rodent diet and water *ad libitum*. The study protocol was approved from Institutional Review Board (IRB) for Animal Research and Ethics committee, Dow University of Health Sciences; Ref no: AR.IRB-013/DUHS/ Approval/2018/014.

Experimental design

Acute, sub-acute and chronic toxicity tests were performed following the corresponding protocols as described by the Organization of Economic Cooperation and Development (OECD) guidelines 425, 451, 453 for testing chemicals (OECD, 2018). The visual observations such as physical appearance, behavioral changes (Abnormal breathing and locomotion, dehydration, eye abnormality, icterus, hyperactivity, diarrhea, writhing, piloerection and restlessness) or any injury or illness including mortality were noted daily throughout the study period (Balogun *et al.*, 2016; Suchantabud *et al.*, 2017). On the final days of treatments i.e. 7^{th} (acute), 14^{th} (sub-chronic) and 90^{th} day (chronic) all animals were weighed, anesthetized via diethyl ether (2-4 ml/jar) using simple 'open-drop' method (Hedrich, 2012). Blood samples were (3-5ml/tube) collected *via* cardiac puncture into non-heparinized tubes for biochemical analysis. Serum samples were evaluated for bilirubin (total, direct and indirect), Alanine aminotransferase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), urea and creatinine using fully automated clinical chemistry analyzer (Abbott automated analyzer, U.S.A). While Serum Alpha fetoprotein (AFP) levels were detected only in chronic toxicity testing samples by ELISA kit, Cat No. E1782Mo (Bioassay technology laboratory, Shanghai, China) following the manufacturer's instructions. Likewise, for histopathology, liver, kidney and spleen in control and treated groups (Acute, subacute and chronic toxicity) were excised and after gross examination of harvested organs small blocks of the tissues were prepared. The tissues were fixed in buffered formalin (10%) for approximately 24 h and then dehydrated in the alcohol dilutions. Afterwards, they are cleared in xylene and then embedded in paraffin. After processing, tissues of 5 µm thickness are sectioned and prepared by using a rotary microtome and dried at 37 °C in an oven overnight. These tissue sections were stained with hematoxylin and eosin (H&E) and processed slides were observed at 40x magnification under a light microscope (Olympus BX41); images were taken by Olympus DP 12 camera (Nigatu *et al.*, 2017). Mortality was observed in treatment groups throughout the study period and LD_{50} (i.e., the dose that kills 50% of animals) was constructed using regression analysis.

Acute toxicity

Prior to the commencement of experiments, mice were fasted for 24h. DCD-684 was administered orally as a single dose of 0.03, 0.15, 0.3, 1.5 and 3ml/kg body weight concentration to mice (n=5/group/ gender) whereas the control group (n=10) received physiological saline (0.9% sodium chloride). All mice are allowed to freely access the food and water and monitored for any indication of toxicity for a period of 24h.

Subacute toxicity

The animals were grouped as explained above in acute study; however, the observations were noted for 14 days and their weights were measured at 0, 7th and 14th day of the study. The similar process was followed at the end of the study as explained earlier.

Chronic toxicity

Mice were randomly assigned into 6 groups as described above, however DCD-684 at 0.3, 9, 12, 18 and 36ml/kg body weight were administered orally for 90 days and control group received physiological saline. Animals were observed and weekly weighed was recorded during the 90 days of treatment period. On the 90th day, the same process was followed as explained earlier. The genotoxicity of DCD-684 and its effect on mRNA expression of cytokines and inflammatory metabolites were also analyzed in this study.

Micronucleus (MN) assay

The genotoxic effect (Micronucleus appearance) induced by DCD-684 was assessed after 90 days of treatment (chronic toxicity). However, cyclophosphamide (CPA, 40 mg/kg) treated animals (positive control) were sacrificed after 24 hours. Mice were euthanized, followed by the collection of bone marrow at the end of the experiment. Hanks's balanced salt solution (500µl) was used to flush the femora of mice. A drop of this mixture was smeared onto a pre-cleaned glass slide, air dried and fixed with methanol (95%) for 10 minutes followed by the staining with 5% Giemsa stain (Krishna and Hayashi, 2000). All slides were randomly coded before examination and micronucleus (MN) were identified and observed in 2000 polychromatic erythrocytes (PCE)/mouse for clastogenic effect of the test agent. The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was also evaluated reflecting cytotoxic effect (Park *et al.*, 2017).

Analysis of pro-inflammatory cytokines and chemokines mRNA by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

The mice livers from control and treated group were collected and a TRIzol method was used to extract the total RNA (Laukova *et al.*, 2018). Its concentration and purity were determined at A260:A280 using the Colibri Micro Volume Spectrophotometer (Titertek Berthold). Complementary DNA (cDNA) was prepared from total RNA (1 mg) using a reverse transcriptase cDNA synthesis kit and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was incorporated as internal control (Thermo Scientific; Life Technologies). Transcriptional gene expression of interleukin-1 Beta (IL-1β), Tumor necrosis factor -Alpha (TNF-α), along with inflammatory chemokines including Cyclooxygenase-2 *(*COX-2), Macrophage Inflammatory Protein 2 (MIP-2) and GAPDH were determined *via* quantitative real-time PCR using a SYBR Green master mix Kit **(**Rotor Gene Q Real-Time PCR Detection System). The PCR conditions followed were: 1 cycle of 95°C (5min), followed by 35 cycles at 95°C (30s), 60 °C (30s) and 72°C (30s). The relative mRNA expression of aforementioned target genes under treated or untreated conditions were calculated by using $2^{\Delta\Delta}$CT comparative threshold (CT) method. The primer sequences used for RT-PCR are presented in table 1.

STATISTICAL ANALYSIS

The experimental data were analyzed using statistical analysis program for social science (SPSS 21). All data are expressed as mean ± standard error of the mean (SEM). Analysis of one-way ANOVA followed by post-hoc Dunnett's comparison test was applied to compare each group with the control. Statistical significance level was set at $p< 0.05$.

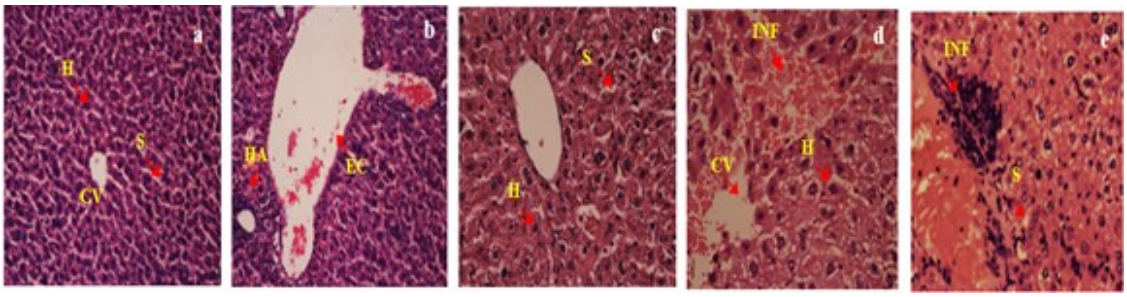
RESULTS

Acute toxicity test

Different concentrations of DCD-684 (0.03-3 ml/kg body weight) neither caused any mortality, morbidity or changed the behavioral responses in mice for up to 7 days (table S1-A). The body weight (male 25.2g ± 0.31 and female 24.7±0.18) in control and treated mice were also similar (fig. S1a-b). Likewise, biochemical parameters (table 2) and histopathological examination of sections derived from liver, kidney and spleen showed similar findings when compared with the control group. The general architecture of the hepatocytes, hepatic sinusoids and central veins were normal. There was no noticeable sign of necrosis, fibrosis and steatosis even at the highest dose of 3 ml/kg body weight (fig. 1a-b). The kidney sections of treated mice also showed normal general structure and glomeruli, proximal and distal convoluted tubules were intact (fig. 2a-b). The spleen from mice treated with DCD-684 also demonstrated normal white pulp containing lymphoid masses and a highly vascular red pulp similar to spleens from control mice (fig. 3a-b).

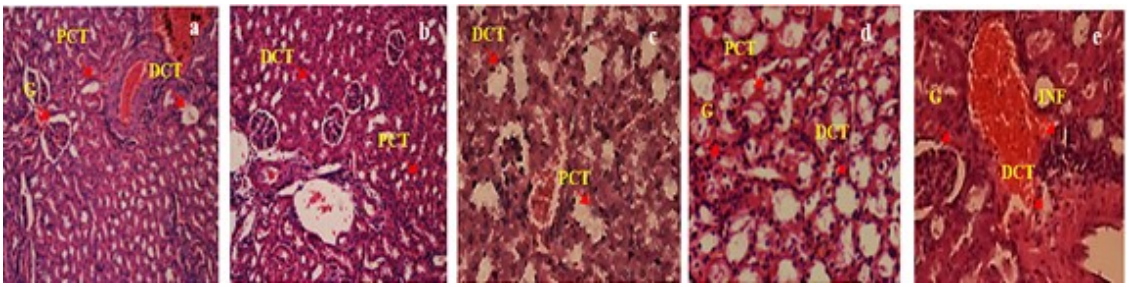
Subacute toxicity test

Daily oral administration of DCD-684 (0.03-3 ml/kg body weight) for 14 days showed no observable side effects and changes in animal behavior, mortality or any adverse effect as mentioned in table S1-B. Body weight revealed no significant changes observed weekly (fig. S2a-b) after DCD-684 treatment as compared to the control group in mice throughout the study duration. Biochemical parameters did not reveal statistically significant differences (table 3) in liver function with slight elevation in ALT, AST and ALP levels. The kidney function parameters (urea and creatinine) also did not exhibit any relevant changes. The histological findings of liver, kidney and spleen of both gender mice showed no modifications in their morphology or architecture as compared to the control group (figs. 1c, 2c and 3c).



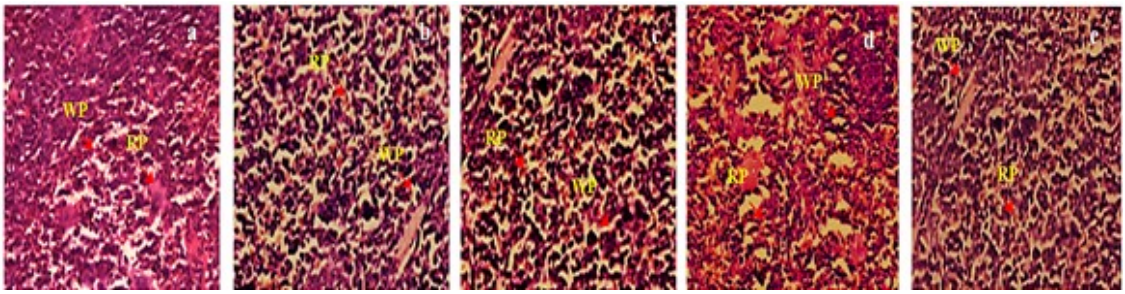
Photomicrographs of hematoxylin and eosin stained slides (40x magnification) of liver sections: (a) Control, sinusoids (S) and hepatocytes arranged as radial plates (H) with prominent central vein (CV), hepatic artery (HA) and endothelial cells (EC) in (b) Acute (7-day duration) and (c) Sub-acute (14-day duration) at 3 ml/kg body weight group while (d) Chronic toxicity at 18 ml/kg body weight and (e) 36 ml/kg body weight group (90-day duration) exhibited mild periportal inflammation (INF) in both male and female mice group (*n=5*).

Fig. 1: Effect of DCD-684 on the liver histology of mice.



Photomicrographs of hematoxylin and eosin-stained slides (40x magnification) of kidney sections: (a) Control, normal glomeruli (G), proximal (PCT) and distal convoluted tubules (DCT) in (b) Acute (7-day duration) and (c) Sub-acute (14-day duration) at 3 ml/kg body weight group and (d) Chronic toxicity at 18 ml/kg body weight while (e) 36 ml/kg body weight group (90-day duration) exhibited interstitial inflammation (INF) with variable sized glomeruli in both male and female mice group (*n=5*).

Fig. 2: Effect of DCD-684 on the kidney histology of mice.



Photomicrographs of hematoxylin and eosin-stained slides (40x magnification) of spleen sections: (a) Control, normal architecture of Red pulp (RP) exhibiting few sinusoids and white pulp (WP) showing lymphoid follicles in (b) Acute (7-day duration) and (c) Sub-acute (14-day duration) at 3 ml/kg body weight, (d) Chronic toxicity groups at 18ml/kg body weight and (e) 36 ml/kg body weight group (90-day duration) in both male and female mice group (*n=5*).

Fig. 3: Effect of DCD-684 on the histopathology of mice spleen.

Table 1: Sequences of different primers used for RT-PCR analysis in chronic toxicity study

Gene	Direction	Sequences (5′ to 3′)
IL-1β	Forward	AATCTCACAGCAGCACATCAA
	Reverse	AGCCCATACTTTAGGAAGACA
TNF-α	Forward	CCCCTCAGCAAACCACCAAGT
	Reverse	CTTGGGCAGATTGACCTCAGC
COX-2	Forward	ACTGAAGCCAGCTCTCTCTT
	Reverse	TTCCTTCTTGGGGTCAGCAC
MIP-2	Forward	GGATTCACCTCAAGAACATC
	Reverse	CACCCTTCTACTAGCACAGTG
GAPDH	Forward	CCATGGAGAAGGCTGGG
	Reverse	CAAAAGTTGTGGATGACC

Table 2: Effect of DCD-684 on serum biochemical parameters in mice during acute toxicity testing

Parameter	Physiological saline (Control)	DCD-684 treated concentration (ml/kg body weight)				
		0.03	0.15	0.3	1.5	3.0
Alanine amino transferase (U/L)	M: 24±4.0 F: 43±5.0	M: 26±4.5 F: 58±4.8	M: 45±4.2 F: 49±3.0	M: 40±2.5 F: 50±7.0	M: 30±4.6 F: 39±4.3	M: 29±9.0 F: 26±11.0
Aspartate transaminase (U/L)	M: 216±6.0 F: 219±2.0	M: 213±6.0 F: 209±4.3	M: 218±8.3 F: 186±6.8	M: 123±2.3 F: 200±3.6	M: 163±4.6 F: 229±4.2	M: 161±4.6 F: 211±5.0
Alkaline phosphatase (U/L)	M: 55±5.0 F: 57±4.2	M: 51±4.3 F: 83±6.0	M: 40±4.8 F: 89±6.3	M: 128±4.1 F: 81±5.3	M: 39±6.2 F: 76±4.0	M: 44±7.2 F: 48±5.0
Serum Direct bilirubin (mg/dl)	M: 0.1±0.0 F: 0.1±0.0	M: 0.1±0.0 F: 0.1 ± 0.0	M: 0.1 ±0.0 F: 0.1±0.0	M: 0.1 ± 0.0 F: 0.1 ± 0.0	M: 0.10 ±0.0 F: 0.10 ±0.0	M: 0.1 ±0.0 F: 0.1 ±0.0
Serum Indirect bilirubin (mg/dl)	M: 0.01±4.0 F: 0.05±5.3	M: 0.02±5.4 F: 0.01±4.0	M: 0.01±4.5 F: 0.08±4.4	M: 0.04±7.2 F: 0.07±4.0	M: 0.02±5.3 F: 0.02±7.2	M: 0.01±6.0 F: 0.04±4.9
Serum Total bilirubin (mg/dl)	M: <0.10±7.2 F: 0.15±7.2	M: 0.12±5.3 F: 0.11±4.0	M: <0.10±2.3 F: 0.18±5.0	M: 0.15±5.0 F: 0.17±4.5	M: 0.12±4.0 F: 0.12±7.3	M: <0.10±7.5 F: 0.14±8.2
Gamma Glutamyl Transferase (U/L)	M: <4.0 ±0.0 F: <4.0 ±0.0	M: <4.0± 0.0 F: <4.0 ± 0.0	M: <4.0 ±1.0 F: <4.0 ± 0.0	M: <4.0 ±0.0 F: <4.0 ± 0.0	M: <4.0 ±0.0 F: <4.0 ± 1.0	M: <4.0 ±0.0 F: <4.0 ±0.0
Urea (mg/dl)	M: 33.57±11.9 F: 36.5 ±10.2	M: 35.7±0.3 F: 34.6±0.15	M: 36.8±0.13 F: 34.2±0.33	M: 32.9±0.21 F: 36.2±0.32	M: 38.2±0.15 F: 36.6±0.32	M: 36.6±0.36 F: 38.5±0.23
Creatinine (mg/dl)	M: 0.32 ±0.18 F: 0.35 ±0.09	M: 0.47±1.1 F: 0.41±1.3	M: 0.39±2.33 F: 0.45±2.13	M: 0.45±2.14 F: 0.47±1.22	M: 0.48±1.44 F: 0.38±1.38	M: 0.45±2.15 F: 0.42±2.18

Table 3: Effect of DCD-684 on serum biochemical parameters in mice during sub-acute toxicity testing

Parameter	Physiological saline (Control)	DCD-684 treated concentration (ml/kg body weight)				
		0.03	0.15	0.3	1.5	3.0
Alanine amino transferase (U/L)	M: 37±4.0 F: 32±5.0	M: 44±6.0 F: 65±4.3	M: 69±4.1 F: 37±2.3	M: 42±5.6 F: 52±4.6	M: 43±5.6 F: 62±4.2	M: 66±4.2 F: 68±4.4
Aspartate transaminase (U/L)	M: 209±4.0 F: 214±6.0	M: 207±5.0 F: 218±7.6	M: 219±4.0 F: 221±4.8	M: 222±4.9 F: 225±7.5	M: 227±8.2 F: 218±7.6	M: 226±8.3 F: 231±7.6
Alkaline phosphatase (U/L)	M: 57±4.0 F: 65±6.3	M: 55±5.8 F: 67±4.0	M: 59±5.8 F: 71±9.3	M: 64±7.3 F: 76±6.9	M: 66±8.2 F: 75±8.0	M: 70±8.3 F: 78±4.9
Serum Direct bilirubin (mg/dl)	M: 0.1±0.0 F: 0.1 ±0.0	M: 0.1±0.0 F: 0.1 ± 0.0	M: 0.1±0.0 F: 0.1± 0.0	M: 0.1± 0.0 F: 0.1± 0.0	M: 0.1±0.0 F: 0.1±0.0	M: 0.1±0.0 F: 0.1±0.0
Serum Indirect bilirubin (mg/dl)	M: 0.01±8.0 F: 0.04±5.3	M: 0.13±4.2 F: 0.05±4.1	M: 0.04±7.3 F: 0.01±4.5	M: 0.03±7.5 F: 0.03±4.8	M: 0.05±8.9 F: 0.05±9.3	M: 0.09±7.8 F: 0.03±7.0
Serum Total bilirubin (mg/dl)	M: 0.11±7.8 F: 0.14±6.0	M: 0.23±7.8 F: 0.15±5.9	M: 0.14±7.8 F: 0.11±7.9	M: 0.13±4.8 F: 0.13±7.8	M: 0.15±7.8 F: 0.15±7.9	M: 0.19±8.9 F: 0.13±4.0
Gamma Glutamyl Transferase (U/L)	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0± 0.0 F: <4.0 ±0.0	M: <4.0± 0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0
Urea (mg/dl)	M: 39.5±11.9 F: 32.5±10.2	M: 38.7±11.5 F: 34.25±9.1	M: 39.7±8.5 F: 34.5±4.4	M: 39.9±3.8 F: 40.0±4.9	M: 40.1±8.3 F: 42.5±2.8	M: 43.8±3.7 F: 44.7±4.9
Creatinine (mg/dl)	M: 0.35±0.11 F: 0.36±0.06	M: 0.37±0.02 F: 0.4± 0.3	M: 0.34±0.05 F: 0.36±0.04	M: 0.40±0.03 F: 0.37±0.05	M: 0.42±0.04 F: 0.39±0.04	M: 0.42±0.06 F: 0.47±0.04

Biochemical parameters were analyzed after the 14 days of daily dose administration of different concentrations to mice groups. Data expressed as Mean ± SEM ($n=5$) and analyzed by one-way ANOVA followed by Dunnett's test. M: Male and F: Female indicates the results for the respective gender in mice. All the values of DCD-684 treated groups showed non-significant (n.s) changes as compared with control mice ($p>0.05$), hence n.s is not shown in the table.

Chronic toxicity test

Physical and behavioral analysis

There were no physical and behavioral alterations in the treated animals at doses 0.3, 9 and 12ml/kg body weight (tableS1-C). However, at higher doses (18 and 36 ml/kg body weight) irregular respiration and abnormal locomotion were observable which were reversed after 2-hrs of administration. At the highest dose (36 ml/kg body weight) of DCD-684, ruffled fur accompanied by lethargy were immediately visible after its administration which was exaggerated during the 90 days' treatment. The body temperature (36-38°C) of mice during the treatment period remained unchanged. The LD_{50} of DCD-684 in mice was 27.5ml/kg body weight (fig. 4). A gradual increase was observed in the body weight with no significant difference in the DCD-684 treatment groups of doses 0.3-18ml/kg body weight which was similar to control group throughout the study time (fig. 5a-b). However, at the 36 ml/kg body weight, animals revealed no progressive weight gain till 28th day (~25g) followed by slight reduction by the 56th day (~24.5g) and 90th day (~24g) which was not statistically significant when compared to control group.

Table 4: Effect of DCD-684 on serum biochemical parameters in mice during chronic toxicity testing

Parameter	Physiological saline (Control)	DCD-684 treated concentration (ml/kg body weight)				
		0.3	9.0	12.0	18.0	36.0
Alanine amino transferase (U/L)	M: 35.6±3.2 F: 43±5.0	M: 41.3±16.2 F: 40.3 ± 11.5	M:40.6±12.5 F: 41.0±1.0	M: 42±2.5 F: 39.6± 14.0	M: 44.6±4.5 F: 43.6±10.7	M: 63.0±7.5* F: 57.3±2.5*
Aspartate transaminase (U/L)	M: 207.0±18.5 F: 206±10.8	M: 212.6±5.0 F: 215.3±48.3	M: 216.0±14 F: 220.3±14.2	M:218.6±38.0 F: 219.3±24.4	M: 246.6±11.7* F: 256.6±29.7*	M: 292.0±55.7** F: 278.0±34.6**
Alkaline phosphatase (U/L)	M: 56.3±4.7 F: 62.3±6.0	M: 55.6±13.6 F: 59.6±12.2	M: 60.6±14.7 F: 57.0±1.1	M: 62.6±13.7 F: 61.0±24.8	M: 86.3±4.7* F: 81.0±23*	M: 94.0±32.1** F: 99.0±66.1**
Serum Direct bilirubin (mg/dl)	M: 0.1± 0.0 F: 0.1± 0.0	M: 0.1± 0.0 F: 0.1± 0.0	M: 0.1± 0.0 F: 0.1± 0.0	M: 0.1± 0.0 F: 0.1±0.0	M: 0.1±0.0 F: 0.1±0.0	M: 0.1± 0.0 F: 0.1± 0.0
Serum Indirect bilirubin (mg/dl)	M: 0.03±0.0 F: 0.01±0.0	M: 0.02±0.0 M: 0.02±0.0	M: 0.03±0.02 F: 0.03±0.01	M: 0.05±0.02 F: 0.04±0.01	M: 0.03±0.02 F: 0.01±0.0	M: 0.03±0.01 F: 0.02±0.03
Serum Total bilirubin (mg/dl)	M: 0.13±0.2 F: 0.11 ± 0.0	M: 0.1±0.01 F: 0.12± 0.02	M: 0.13± 0.03 F: 0.13± 0.01	M: 0.15±0.07 F: 0.14±0.08	M: 0.13±0.0 F: 0.11±0.0	M: 0.13±0.01 F: 0.12±0.0
Gamma Glutamyl Transferase (U/L)	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0	M: <4.0±0.0 F: <4.0±0.0
Alpha Feto Protein (ng/ml)	M: 0.34±0.01 F: 0.33±0.23	M: 0.34±0.36 F: 0.351±0.12	M: 0.38±0.3 F: 0.38±0.4	M: 0.39±0.41 F: 0.40±0.10	M: 1.302±0.07* F: 1.204±0.01*	M: 1.808±0.04** F: 1.68±0.16**
Urea (mg/dl)	M: 46.57±11.9 F: 49.5±10.2	M: 45.7±1.56 F: 48.25±5.1	M: 49.7±2.5 F: 49.5±5.4	M: 49.9±2.2 F: 50.0±4.1	M: 50.1±2.2 F: 49.5±4.1	M: 58.7±3.81* F: 57.7±4.8*
Creatinine (mg/dl)	M: 0.41±0.12 F: 0.35±0.04	M: 0.35±0.02 F: 0.4± 0.28	M: 0.35±0.0 F: 0.33± 0.01	M: 0.40±0.06 F: 0.38±0.13	M: 0.41±0.02 F: 0.39±0.1	M: 0.48±0.01 F: 0.53±0.02

Biochemical parameters were analyzed after the 90 days of daily dose administration of different concentrations to mice groups. Data expressed as Mean ± SEM ($n=5$) and analyzed by one-way ANOVA followed by Dunnett's test. M: Male and F: Female indicates the results for the respective gender in mice. Asterisks indicate significant values (*$p < 0.05$ and **$p < 0.01$), whereas other values were non-significant in the table.

Table 5: Frequency of micronucleus in the presence and absence of DCD-684 in mice erythrocytes

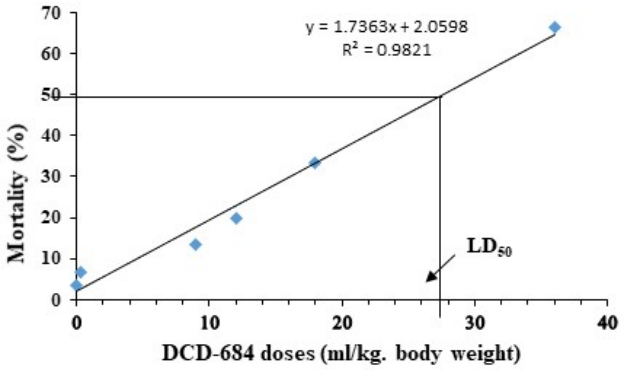
Treatments		Gender	PCE	MN-PCE	MN-PCE/PCE (‰)	PCE/NCE Mean ± SEM
Control	CPA (40mg/kg)	Male	2000	77	38.5±1.0*	0.42 ± 0.02
		Female	2000	81	40.5±1.0*	0.43 ± 0.02
	Physiological saline	Male	2000	6	3.0±1.0	0.86± 0.02
		Female	2000	3	1.5±0.0	0.85± 0.01
DCD-684 Treated Groups (ml/kg body weight)	0.3	Male	2000	4	2.0±1.0	0.83 ± 0.01
		Female	2000	5	2.5±2.0	0.84 ± 0.02
	9.0	Male	2000	6	3.0±1.0	0.82± 0.02
		Female	2000	5	2.5±2.0	0.79± 0.01
	12.0	Male	2000	7	3.5±2.0	0.79± 0.01
		Female	2000	6	3.0±1.0	0.78 ± 0.02
	18.0	Male	2000	8	4.0±1.0	0.77± 0.02
		Female	2000	10	5.0±3.0	0.75± 0.02
	36.0	Male	2000	11	5.5±1.5	0.75± 0.02
		Female	2000	13	6.5±3.0	0.77± 0.01

Data are expressed as Mean ± SEM ($n= 5$/sex) as compared to control. *$p<0.05$ Rate (‰), Cyclophosphamide (CPA), Digas Colic Drops (DCD), Micronucleated Polychromatic Erythrocyte (MN-PCE), Normochromatic Erythrocyte (NCE) and Polychromatic Erythrocyte (PCE)

Biochemical and histological analysis

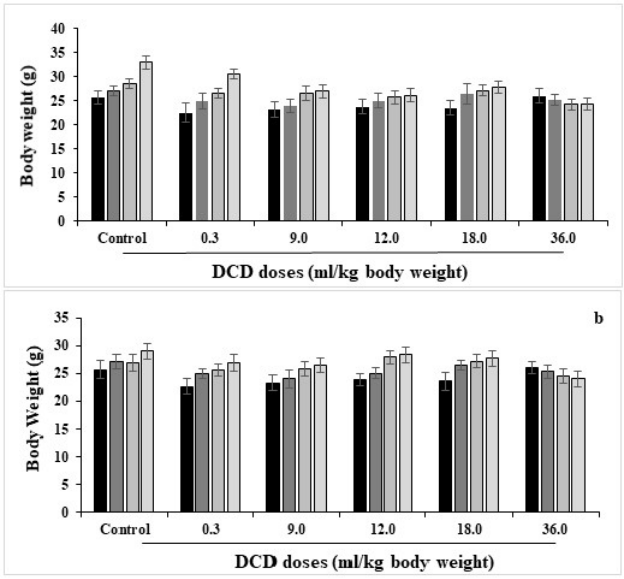
The oral administration of DCD-684 at 0.3, 9 and 12 ml/kg body weight after 90th day of the treatment revealed no significant changes in the liver and kidney functions as well as in serum AFP levels (table 4). Likewise, there were no changes in the hepatic and renal architecture as compared to the control group in both genders. However, at 18 ($p<0.05$) and 36ml/kg body weight ($p<0.01$) AST, ALP and AFP levels were increased significantly. While at 36 ml/kg body weight, ALT level was also notably raised ($p<0.05$). These findings clearly correlated with the mild and moderate periportal inflammation at 18 and 36 ml/kg body weight with few dilated sinusoids in liver sections of both male and female mice (fig. 1d-e)

respectively. At 36 ml/kg body weight in both genders, serum urea levels were raised (p<0.05) and renal parenchyma showed moderate amount of interstitial inflammation with variable sized glomeruli (fig. 2e). However, in the splenetic tissues there was no sign of hemosiderin laden macrophages, malignancy or any pathological changes in all the treatment groups (fig. 3d-e).



The X-axis represents different concentrations of DCD-684 including 0.3, 9, 12, 18 and 36 ml/kg body weight, respectively while the Y-axis represents the mortality of the mice in percent. LD: Lethal Dose.

Fig. 4: The LD_{50} of DCD-684

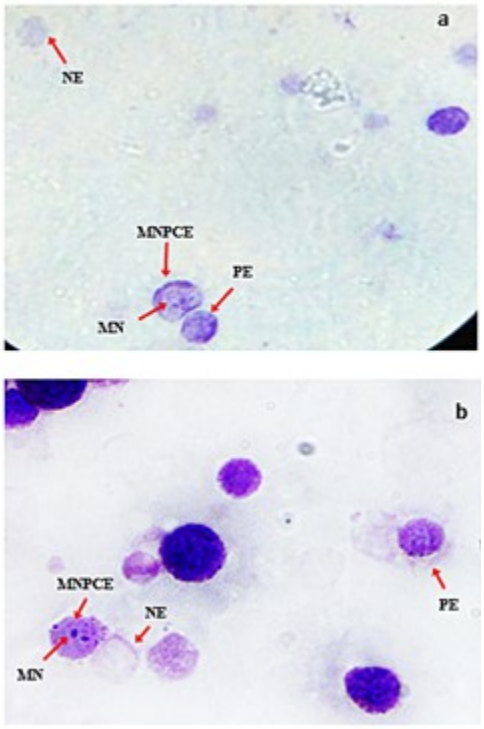


Body weight after DCD-684 treatment: **(a)** Male and (**b)** Female mice (n = 5/sex/group) for 1st day (●), 28th day (●), 56th day (●) and 90th day (●). Control (C) and treated groups received physiological saline or different concentrations of DCD-684 (ml/kg body weight). Values of treated and control groups (p > 0.05) were non-significant (n.s.) and hence not shown

Fig. 5: Effect of DCD-684 on body weight of mice during chronic toxicity study.

Micronucleus (MN) assay

On the 90th day of DCD-684 administration (0.3-36ml/kg body weight) the number of Micro nucleated Polychromatic Erythrocyte (MNPCE)/2000 and Polychromatic Erythrocyte (PCE) per animal in both male and female mice were evaluated (table 5). The frequency of micronuclei increased dose-dependently from 2-6% which lies within the range of control groups. On the contrary, cyclophosphamide used as a positive control induced significant rise of 38-40% (p<0.05) of MNPCE as compared to the control group (fig. 6). Additionally, the polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) ration in the presence of DCD-684 treated groups was within the range of 0.83-0.77 similar to the control values (0.85-0.86). However, a significant decline of 0.41-0.43 was evident in the presence of cyclophosphamide.

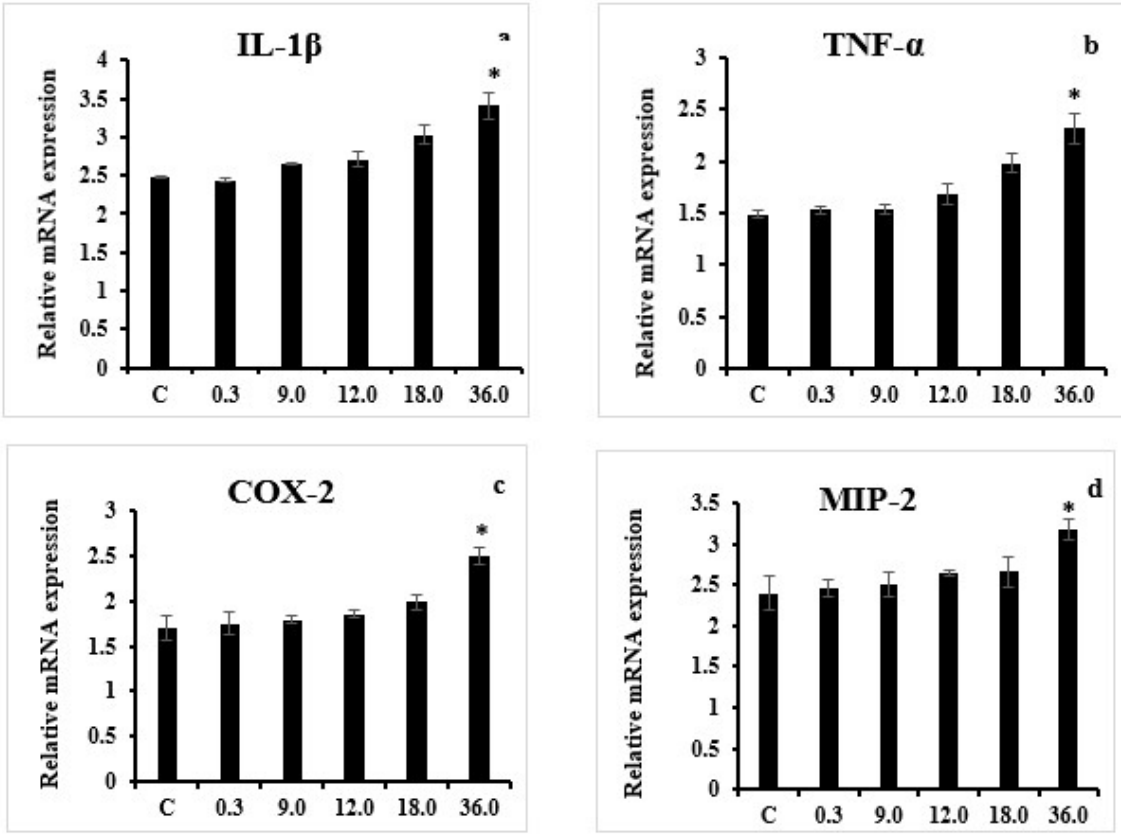


Photomicrographs of Giemsa-stained bone marrow cells of mice treated with (a) DCD-684 at 36 ml/kg body weight dose and (b) cyclophosphamide showing: Polychromatic erythrocyte (PE), Normo-chromatic erythrocyte (NE) and Micro-nucleated polychromatic erythrocyte (MNPCE), Micronucleus (MN) at 100x magnification.

Fig. 6: Micronucleus assay in mice bone marrow

mRNA expression of pro-inflammatory cytokines and chemokines

The transcriptional (mRNA) expression of targeted genes i.e. IL-1β, TNF-α, COX-2 and MIP-2 on 90th day of DCD-684 treatment (0.3-36ml/kg body weight) is presented in fig. 7a-d. Overall consistent expression of all the targeted genes was observed from 0.3-18ml/kg body weight. Conversely, at highest dose (36ml/kg body weight) the mRNA expression of IL-1β, TNF-α, MIP-2 and COX-2 was significantly raised (p < 0.05) by 1.3-1.6-fold, respectively.



mRNA expressions represented by a bar diagram of (a)IL-1β, (b) TNF-α, (c) COX-2 and (d) MIP-2 normalized to the housekeeping GAPDH gene on the y-axis while the Control (C) and treatment groups of different concentration of DCD-684 (ml/kg body weight) for 90-days oral administration on the x-axis. The data calculated using $2^{-\Delta\Delta Ct}$ indicate fold change in transcriptional gene expression ± SEM (n = 5/sex) *p<0.05.

Fig. 7: The effect of DCD-684 on mRNA expression rate of primary pro-inflammatory cytokines in chronic toxicity study.

SUPPLEMENTARY MATERIAL

Table S1-A: Effect of DCD-684 on health assessment of mice during acute toxicity testing

S. No.	Sign & Symptoms	Control	Concentration (ml/kg body weight)				
			0.03	0.15	0.3	1.5	3.0
1.	Abnormal breathing	Normal	Normal	Normal	Normal	Normal	Normal
2.	Abnormal locomotion	Normal	Normal	Normal	Normal	Normal	Normal
3.	Dehydration	No	No	No	No	No	No
4.	Diarrhea	No	No	No	No	No	No
5.	Eye abnormality	Normal	Normal	Normal	Normal	Normal	Normal
6.	Head tilt	No	No	No	No	No	No
7.	Hyperactivity	No	No	No	No	No	No
8.	Hypothermia	No	No	No	No	No	No
9.	Icterus	No	No	No	No	No	No
10.	Lethargy	No	No	No	No	No	No
11.	Paralysis	No	No	No	No	No	No
12.	Paresis	No	No	No	No	No	No
13.	Prenuptial or vaginal discharge	No	No	No	No	No	No
14.	Ruffled fur	No	No	No	No	No	No
15.	Tremor	No	No	No	No	No	No

Table S1-B: Effect of DCD-684 on health assessment of mice during sub-acute toxicity testing

S. No	Sign & Symptoms	Control	Concentration (ml/kg body weight)				
			0.03	0.15	0.3	1.5	3.0
1.	Abnormal breathing	Normal	Normal	Normal	Normal	Normal	Normal
2.	Abnormal locomotion	Normal	Normal	Normal	Normal	Normal	Normal
3.	Dehydration	No	No	No	No	No	No
4.	Diarrhea	No	No	No	No	No	No
1.	Eye abnormality	Normal	Normal	Normal	Normal	Normal	Normal
2.	Head tilt	No	No	No	No	No	No
3.	Hyperactivity	No	No	No	No	No	No
4.	Hypothermia	No	No	No	No	No	No
5.	Icterus	No	No	No	No	No	No
6.	Lethargy	No	No	No	No	No	No
7.	Paralysis	No	No	No	No	No	No
8.	Paresis	No	No	No	No	No	No
9.	Prenuptial or vaginal discharge	No	No	No	No	No	No
10.	Ruffled fur	No	No	No	No	No	No
11.	Tremor	No	No	No	No	No	No

Mice were observed for a period of 14 days. All animals were active, healthy and with no signs of colic drops induced changes throughout the study period.

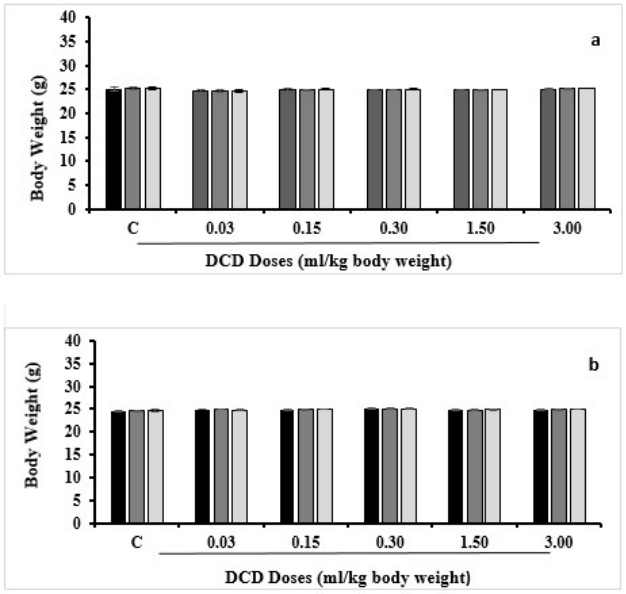
Table S1-C: Effect of DCD-684 on health assessment of mice during chronic toxicity testing

S. No.	Sign & Symptoms	Control	Concentration (ml/kg body weight)				
			0.3	9.0	12.0	18.0	36.0
1	Abnormal breathing	Normal	Normal	Normal	Normal	+	++
2	Abnormal locomotion	Normal	Normal	Normal	Normal	+	++
3	Dehydration	No	No	No	No	No	No
4	Diarrhea	No	No	No	No	No	No
5	Eye abnormality	Normal	Normal	Normal	Normal	Normal	Normal
6	Head tilt	No	No	No	No	No	No
7	Hyperactivity	No	No	No	No	No	No
8	Hypothermia	No	No	No	No	No	No
9	Icterus	No	No	No	No	No	No
10	Lethargy	No	No	No	No	No	+
11	Paralysis	No	No	No	No	No	No
12	Paresis	No	No	No	No	No	No
13	Prenuptial or vaginal discharge	No	No	No	No	No	No
14	Ruffled fur	No	No	No	No	No	+
15	Tremor	No	No	No	No	No	No

"+" = presence of signs and symptoms
"++" = presence of sign and symptoms with certain intensity on the basis of duration and severity of symptoms.
The experimental animals did not show any sign of abnormal physical and behavioral changes, distress and/or any other symptoms of toxicity at the 0.3, 9.0 and 12.0 ml/kg body weight. These results were quite similar as observed in control group. However, at the dose of 18.0 ml/kg body weight of the DCD-684 caused irregular respiration and abnormal locomotion which disappeared after 2hrs of dose administration. At the highest dose of 36.0 ml/kg body weight of DCD-684, hypo-activity, irregular respiration and abnormal locomotion was noted immediately after administration of the dose in addition to ruffled fur and lethargy, which were persistently exhibited and exaggerated during the 90 days' treatment.

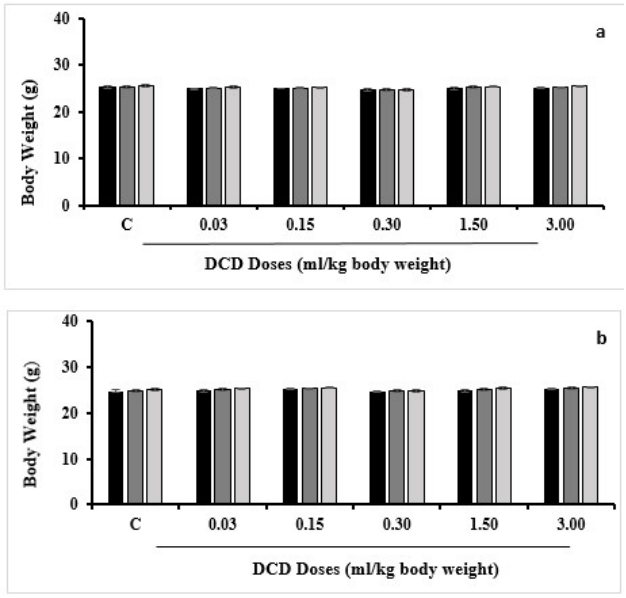
DISCUSSION

Since ancient times, herbal remedies incorporating medicinal plants continued to improve and satisfy the healthcare needs of both human and animals. According to WHO ~80% of the world's population prefer to use traditional remedies as their major health care resource, a main part of which are derived from medicinal plants (Khan *et al.*, 2013). Polyherbal formulations are excessively utilized in developed countries as a remedy for different types of diseases (Ishtiaq *et al.*, 2017). Their extensive use against chronic ailments may lead to adverse effects predominantly leading to renal and hepatic toxicities emphasizing that doses exceeding than the therapeutic dose should be avoided (Liyanagamage *et al.*, 2020).



Body weight after DCD-684 treatment: (a) Male and (b) Female mice (n=5/sex/group) for 1st day (●), 4th day (●) and 7th day (●). Control (C) and treated groups received physiological saline or different concentrations of DCD-684 (ml/kg body weight). Values of treated and control groups (p>0.05) were non-significant (n.s.) and hence not shown.

Fig. S1: Effect of DCD-684 on body weight of mice during acute toxicity study.



Body weight after DCD-684 treatment: (a) Male and (b) Female mice (n=5/sex/group) for 1st day (●), 7th day (●) and 14th day (●). Control (C) and treated groups received physiological saline or different concentrations of DCD-684 (ml/kg body weight). Values of treated and control groups ($p > 0.05$) were non-significant (n.s.) and hence not shown.

Fig. S2: Effect of DCD-684 on body weight of mice during sub-acute toxicity study.

Infantile colic is an ailment affecting a huge population, but its etiology is still not completely elucidated, therefore, its treatment is a little ambiguous (Waddell, 2013). In Pakistan, DCD-684 is popular in mothers for the management of infantile colic pain in infants and other GI disturbances. However, evidence-based studies related to its safety profile are not known. Despite being widely claimed to be naturally safe and immunoprotective, this polyherbal formulation needs to be validated by scientifically proven tests and guidelines for identifying its toxicological index and doses. The present study was designed to analyze the acute, subacute, chronic and genotoxicity of DCD-684 in male and female albino mice.

Acute toxicity test evaluates the undesirable and harmful effects that appear within a small interval of time right after single dose administration of a test substance. It is applied mainly on to the rodents generally performed earlier in the development of a new product, formulation or chemical agent to acquire evidences about its possible toxicological effects (Chambers, 1987). In acute and sub-acute toxicity testing, single and repeated oral administration of DCD-684 at all the doses showed no dose-related alterations in body weights, mortality or clinical signs for both mice genders throughout the study period. Changes in the mean body weight is a critical index for the evaluation of toxicity of any drug substance and a decline of greater than 10% of the body weight is taken as a detrimental effect of that test substance (Vahalia *et al.*, 2011; Tan *et al.*, 2008; Féres *et al.*, 2006). In the chronic toxicity study, the outcomes revealed a non-significant difference in the body weights of mice administered 0.3, 9, 12 and 18ml/kg body weight of DCD-684. However, 36ml/kg body weight of DCD-684 did not show any progressive weight gain with respect to control group. These findings reflect that DCD-684 does not affect the appetite of mice suggesting the satisfactory health status of animals during 90 days' treatment of DCD-684 at all the doses tested. Behavioral studies revealed irregular respiration and abnormal locomotion at 18 ml/kg body weight accompanied with lethargy and ruffled fur in mice at 36ml/kg body weight which could possibly indicate drug-induced interstitial lung disease (DILD) or pulmonary drug toxicity via direct or indirect drug effect. Because lungs could also possibly act as a main metabolic site for several drugs or one of its primary metabolites (Camus and Rosenow, 2004; Flieder and Travis, 2004; Nemery *et al.*, 2001) causing progressive dyspnea (shortening of breath) and reduced physical activity as a sign of chronic form of the disease (Ganguli and Pirmohamed, 2006). Ruffled fur in mice is an indication of poor health status, if noticed along with signs such as loss of body condition or dehydration (Foltz et al. 1999). These signs were also observed at highest tested dose i.e., 36 ml/kg body weight except dehydration although no such effects were noted in control and low dose treatment groups. However, the recognition of DILD is difficult to recognize and not easily distinguished on

examination because of its nonspecific clinical, radiological, and histological findings (Nemery *et al.*, 2001; Schwaiblmair *et al.*, 2012). Therefore, further investigations are required to strongly conclude if DCD-684 causes any pulmonary drug toxicity.

Liver and kidney are vital organs of the body having essential role in metabolism, elimination and detoxification of drugs or other agents (Vaidya *et al.*, 2010). They are the main target organs of many drugs including some nonsteroidal anti-inflammatory drugs, aminoglycoside antibiotics, anticancer drugs, etc. Thus oral administration of these drugs more likely induce adverse effects at the organ level leading to nephrotoxicity. Another primary cause for nephrotoxicity nephrotoxicity are free radicals generated in the kidney cortex leading to dysfunction of renal proximal tubule cells. (Worasuttayangkurn *et al.*, 2012; Sharma *et al.*, 2020). Serum levels of four important enzymes i.e. ALT, AST, ALP and GGT are generally considered as clinical biochemical markers allied with liver failure or injury. Serum levels of ALP identified to be elevated as a result of biliary obstruction (Ozdil *et al.*, 2010) while increased levels of ALT and AST might indicate drug induced liver injury (Mukinda and Syce, 2007; Han *et al.*, 2011; Limdi and Hyde, 2003). ALT and AST are mainly produced by liver and contribute in gluconeogenesis by inducing the catalytic transfer of amino groups from aspartic acid or alanine to ketoglutaric acid to produce oxaloacetic acid and pyruvic acid, respectively. An increase in these enzymes levels might arise from any liver injury or hepatocellular toxicity (Adedapo *et al.*, 2004). In addition, alkaline phosphatase (ALP) is cytoplasmic in nature which enters into the circulatory system on the occasion of liver injury due to the changes occur in membrane permeability. Therefore, elevated levels of serum ALP reveal acute liver injury and hepatocellular inflammatory disorders (Zimmerman *et al.*, 1965). A reduction in serum bilirubin depicts reduced synthetic function, which can also be evident in liver damage or related diseases, while Gamma-glutamyl transferase (GGT) act as a modulator of cellular oxidative stress by regulating oxidized glutathione levels which is the major cell antioxidant (Irie *et al.*, 2012). Serum Alpha Feto Protein (AFP) is a biomarker indicator for hepatocellular carcinoma, usually increased in various non-neoplastic hepatic disorders such as acute liver damage with extensive necrosis (Saleem *et al.*, 2018). Increased serum AFP levels seems to be related with hepatic regeneration followed by the liver injury (Yang *et al.*, 2002). Experimentally, it is observed that serum AFP levels are increased less in mice in case of toxic liver injury (Watanabe *et al.*, 1976). Significant increase in serum ALT levels at 18 ml/kg body weight while AST, ALP and AFP levels showed significant elevation at 36 ml/kg body weight treated group of DCD-684 in the chronic study for both genders which reflect the severe treatment related inflammatory changes and acute liver damage. These findings of biochemical indices correlated with the histopathological examinations in which the chronic administration revealed mild and moderate interphase inflammation in the liver sections at 18 and 36 ml/kg body weight treatment groups. However, the acute and sub-acute study of DCD-684 showed no significant changes in the liver biomarkers and histology of both mice genders having no sign of necrosis, fibrosis and steatosis even at the highest dose of 3 ml/kg body weight. This led us to suggest that all the doses of DCD-684 which are < 18 ml/kg body weight do not instigate the liver toxicity leading to hepatic necrosis in 90-day repeated dose study. Serum urea and creatinine levels are generally applied in clinical biochemistry test for the assessment of renal functionality (Ramaiah, 2011) and considered as main indicators of kidney damage (Loha *et al.*, 2019). However, the creatinine levels are sensitively considered as an indicator of renal malfunction only when the flow rate of the glomerular filtrate has decreased below 50% (Kaid *et al.*, 2019). The serum urea was increased only in 36 /kg body weight DCD-684 treatment group indicating renal dysfunction which was further confirmed by the kidney histology revealing renal inflammation at 36ml/kg body weight in both male and female while creatinine levels were normal at all the doses. Taken together the results revealed that DCD-684 showed no such alterations in renal functional indexes or toxicity at the dose of <36ml/kg body weight thus supporting the wide safety margin of DCD-684 when administered chronically.

The mammalian *in vivo* micronucleus assay is a popular and useful method for genotoxicity testing and bio-monitoring of different chemicals and substances (Pedersen *et al.*, 2009; Sommer *et al.*, 2020). This method allows the identification of both clastogenic (chromosome disruption) and aneugenic (chromosome lagging due to damage of mitotic spindle apparatus) effects by chemical agents (Norppa and Falck, 2003). When erythroblast of a bone marrow matures into a polychromatic erythrocyte, its main nucleus is extruded. In a damaged cell, any micronucleus produced as a consequence may remain behind in anucleated cytoplasm which can be visualized easily in these cells due to absence of main nucleus. The significant rise in the frequency of micro-nucleated polychromatic erythrocytes (MNPCE) followed by the treatment in the subjected animal is a clear sign of induced chromosomal disruption. Additionally, the bone marrow toxicity induced by mutagens is also indicated by the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) (Suzuki *et al.*, 1989). DCD-684 showed normal range of MN-PCE/PCE ratio as well as PCE/NCE as compared to the control values for all the treated doses. Therefore, under our experimental conditions, all the aforementioned doses of DCD-684 did not exhibit clastogenic/aneugenic activity and hence it is non-genotoxic.

The primary pro-inflammatory cytokines, comprising tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) are released sequentially and amplify cellular activation and production of pro-inflammatory cytokines and chemokines (Tarrant, 2010). The TNF-α is a pleiotropic cytokine which plays a significant role in liver injury by inducing hepatocyte apoptosis. It is also involved in the development of the immune system, cell survival signaling pathways and proliferation (Zhao *et al.*, 2020). Another pro-inflammatory cytokine, MIP-2 is produced in response to cell injury and plays a dual role in the development of liver diseases by mediating liver inflammation and regeneration at higher and low concentrations respectively. (Qin *et al.*, 2017). Moreover, cyclooxygenase-2 (COX-2) is highly inducible gene in murine macrophages stimulated by IL-1ß and TNF-α (Feng *et al.*, 1995). Changes in mRNA expression of targeted pro-inflammatory genes including IL-1β, TNF-α, MIP-2 and COX-2 in liver tissues were determined using SYBR green real time PCR after normalization to internal control GAPDH mRNA levels. $2^{-\Delta\Delta Ct}$ is a log-fold-change indicate "X-fold increase or decrease" of target gene with respect to control group. Over all, very consistent expression of targeted genes i.e. IL-1B, TNF-a, MIP-2 and COX-2 was perceived in treatment groups of 0.3, 9, 12 and 18 ml/kg body weight comparable to control group. However, at the highest dose of 36 ml/kg body weight the mRNA expression of IL-1β, TNF-α, MIP-2 and COX-2 was raised by 1.3-1.6-fold significantly. Thus, it can be suggested that DCD-684 at 0.3, 9, 12 and 18 ml/kg body weight were non-toxic while the 36 ml/kg body weight of DCD-684 caused slight increase in mRNA expression which is also well correlated with the biochemical and histopathological examination. Collectively, our data strongly demonstrated that DCD-684 can be safely used orally at a dosage <18 ml/kg body weight in mice exhibiting no toxicological interaction between the plant components constituting the polyherbal formulation. Therefore, this study could be a progressive step towards the human clinical and pre-clinical studies.

CONCLUSION

Our findings imply that oral use of polyherbal DCD-684 is devoid of hepato- and renal- toxicities, non-genotoxic and non-toxic for long term use without any detectable undesirable effects in male or female mice. However, at extremely high doses (18 and 36 ml/kg body weight/day) administered for 90 days, mild hepato-and renal toxic effects were evident without any sign of necrosis or fibrosis but with no genotoxicity. Therefore, toxicological profile of DCD-684 showed wide margin of safety for the relief of infantile colic.

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