ORIGINAL ARTICLE

Evaluation of the sub-chronic oral toxicity of siddha herbo-mineral formulation Kalsiwin tablet

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Abstract

Introduction: To determine the safety index of Siddha herbo-mineral formulation Kalsiwin tablet on chronic oral administration, the sub-chronic oral toxicity studies were carried out by measuring its no-observed-adverse-effect level (NOAEL) and maximum tolerated dose (MTD) in rats. **Materials and Methods:** The sub-chronic oral toxicity of Kalsiwin was evaluated as per Organization for Economic Cooperation and Development 408 Guidelines in either sex of Wistar rats. Kalsiwin was administered at two dose level one is 45 mg/kg/day as rat dose equivalent to 500 mg adult clinical dose, the other one is 90 mg/kg/day is double the dose level of normal clinical dose to determine MTD. In a sub-chronic study, Kalsiwin was administered 90 days. Mortality, observational, behavioral changes, feed and water consumption, hematological, biochemical parameters, organ weight, histopathology of the liver, kidney, and intestine were observed during the study period. **Results:** In the sub-chronic administration of Kalsiwin at the therapeutic dose level did not show any severe toxicity symptoms. Higher dose level (90 mg/kg) showed significant changes in the liver biochemical markers and histological changes in the liver, kidney, and intestine. Furthermore, X-ray studies showed renal calculi formation in two out of three male rats treated with a higher dose level of Kalsiwin. **Conclusion:** The NOAEL of Kalsiwin was 500 mg/day of human therapeutic dose and MTD was less than 1000 mg/day. This study suggested that 500 mg/day adult dose of Kalsiwin is safer on chronic use.

Key words: *Cissus quadrangularis,* Kalsiwin, sangu parpam, sub-chronic toxicity

INTRODUCTION

Siddha is one of the widely used plant-based alternative systems of the Indian traditional medicinal system.[1] About 80% of the worldwide population particularly in developed countries using natural medicine reported by WHO, because they are cheap, available, and consider safe than allopathic medications.[2,3] In recent days, attention toward herbal medicine was increased due to its safety and wide acceptability. Moreover, it has been provided excellent clinical results with fewer adverse effects than synthetic medicines.[4,5] Before the transformation of traditional medicine into the acceptable herbal formulation significant toxicological data of those formulations are much needed. However, the toxicological data of the herbal formulations are rarely been reported. Hence, there is a need for various documented systemic toxicological screenings to explore the possible toxic effects of herbal formulations, to enhance its acceptability.

Kalsiwin is the shell-based calcium supplement of SKM Siddha and Ayurvedha Company, Tamil Nadu, India, indicated to prevent osteoporosis and to maintain the strength of bones. It is the combined herbo mineral Siddha formulation of Sangu parpam and *Cissus quadrangularis* extract. *C. quadrangularis* helps in reducing pain, swelling, and fracture mobility and accelerates the healing of

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fracture jawbones.[6] Ayush practitioners in India clinically prescribing Kalsiwin widely to prevent osteoporosis and in the management of bone-related problem. However, there is little knowledge about its toxicity. Therefore, it is necessary to evaluate its safety profile. As a part of the safety evaluation of Kalsiwin, sub-chronic oral toxicity studies were conducted to investigate the possible side toxic effect after 90 days of repeated oral dosing of Kalsiwin in Wistar rats. This study was carried out by the Organization for Economic Cooperation and Development (OECD) test guidelines, 408.[7]

MATERIALS AND METHODS

Components of Kalsiwin

Kalsiwin (Batch number - KLS 02) was provided by SKM Siddha and Ayurvedha Company (India) Pvt. Ltd. (Tamil Nadu, India). It contains Sangu parpam (67%) and *C. quadrangularis* extract (17%) formulated as tablets. The practicing clinical adult dose of Kalsiwin is 500 mg/day, it was converted in to rat dose 45 mg/kg based on body surface area[8] to evaluate the no-observed-adverse-effect level (NOAEL) and 90 mg/kg which is double the therapeutic dose used to detect the maximum tolerated dose (MTD) in rats.

Experimental Animals

The colony inbred mature both male and female albino Wistar rats (2–3-month-old), weighing 250–300 g were included and obtained from the Central Animal House of Swamy Vivekanandha College of Pharmacy, Namakkal, Tamil Nadu, India. The animals were kept under standard environmental conditions of 12/12 h light/dark rhythm, maintained under controlled room temperature (23 ± 2°C) and relative humidity of 60% ± 10% in polypropylene cages. They were fed with standard pellet diet and water *ad libitum*. Totally 18 rats were housed each cage contained 3 rats of individual sex with bedding of husk. The cages were cleaned daily by changing the husk bedding. All the animals were acclimatized to the standard laboratory condition for 7 days before the start of the study. The experimental procedures were followed under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi. The experimental protocol was approved by the Institutional Animal Ethics Committee of Swamy Vivekanandha College of Pharmacy, Namakkal, Tamil Nadu, India (SVCP/IAEC/RD/1/13/2019).

Sub-chronic Toxicity Studies

The sub-chronic toxicity study of Kalsiwin was performed according to OECD guideline No. 408[7] with slight modification. Rats were randomly divided into three groups of 6 animals each. Group-I received 0.5 ml of 1% CMC solution, Group-II received 45 mg/kg of Kalsiwin (500 mg/day clinical therapeutic dose level), and Group-III received 90 mg/kg of Kalsiwin (1000 mg/day higher therapeutic dose level). The doses of Kalsiwin were freshly prepared daily in 1% CMC solution and administered by oral gavage for 90 days.

Clinical Signs, Body Weight, Feed, and Water Intake

Daily all the animals were observed for clinical signs and mortality. The weight and behavioral changes of each rat were weekly recorded throughout the study period. The consumption of feed and water was also observed daily. The clinical signs include changes in the skin, fur, eyes, mucous membranes, secretions and excretions, automatic activity and changes in gait. Also, behavioral examination such as posture and response to various stimuli were carried out. These behavioral examinations included the stereotypes (i.e. excessive grooming, repetitive circling) or bizarre behavior (i.e. self mutilation), assessment of grip strength and motor activity were carried out periodically every week after administration of the drug.

Hematological Analysis

On the 91st day, the blood samples were collected by a retro-orbital puncture in ethylene diamine tetra acetate container for hematological analysis by established procedures using automated YUMIZEN H500 hematology analyzer. The estimated parameter includes hemoglobin (Hb), erythrocyte count (RBC), total and differential leukocyte counts (WBC), platelet count, mean corpuscular volume, mean corpuscular Hb, and mean corpuscular Hb concentration.

Biochemical Analysis

On the 91st day, the serum separated from a blood sample collected into the plain tube and centrifuged at 3500 rpm for 10 min for the assay of biochemical parameters including alkaline phosphatase (ALP), alanine aminotransferases, aspartate transaminases, total protein, albumin, globulin, urea, creatinine, and total cholesterol by established procedures using automated VITROS 5.0/FS analyzer.

X-ray Analysis

Rats were anesthetized with ketamine 125 mg/kg and xylazine 10 mg/kg i.p. X-ray analysis of the anesthetized rat was carried for the occurrence of renal calculi measurement in the treatment groups.

Histopathological Analysis

After weighing, liver, kidney, and intestine were preserved in 10% formalin at least 24 h, dehydrated in a graded alcohol series (70, 90, 95, and 100%), embedded in paraffin, 4–5 μm thick sections were cut and stained with hematoxylin-eosin for microscopic examination and interpretation.

Statistical Analysis

Results were presented as mean ± standard error of mean of sample replicates (n = 6). Raw data were analyzed by using one-way analyses of variance, followed by *Post hoc* Dunnett's test using SPSS V.17. $P < 0.05$ was established to be statistically significant.

RESULTS

Effect of Kalsiwin on Body Weight Changes

Normal body weight gains were observed in all the treatment groups. No significant differences were observed between the normal control and Kalsiwin treatment groups. However, visually slight increases in body weight of Kalsiwin treated groups in comparison with normal control animals [Table 1].

Effect of Kalsiwin on Feed Intake and Water Intake

There was no feed intake difference among all the treatment groups. Final water intake was higher in Group-III, and slightly less in Group-II in comparisons with normal control [Table 2].

Effect of Kalsiwin on Behavior and Observational Parameters

The changes in clinical signs such as skin color, fecal consistency, gait analysis, urine analysis, sensory responses, animal behavior abnormalities, and neuromuscular coordination have been used as an indicator of adverse effect. Since no remarkable changes were observed in observational and behavior parameters in Kalsiwin treated rats as compared to the control group. Loose stools were identified in the Group-III treatment on 61st and 91st day. It can be inferred that Kalsiwin was nontoxic at the administered dose up to 45 mg/kg in rats [Table 3].

Effect of Kalsiwin on Hematological Parameters

Sub-chronic administration of Kalsiwin in the hematological parameter showed no significant variation in erythrocytes, hb, leukocytes, and platelet content as compared to the control group [Table 4].

Effect of Kalsiwin on Biochemical Parameters

No, statistically significant differences were recorded in serum hepatic biochemistry parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), ALP, and total bilirubin. However, moderate significant ($P < 0.05$) changes were observed in gamma-glutamyl transferase (GGT), serum albumin, and globulin in Group-III as compared to the normal control. There is no significant difference in the serum total cholesterol, urea, and creatinine level in the Kalsiwin treatment groups as compared to the normal control [Table 5].

Effect of Kalsiwin on Mortality and Relative Organ Weight

No mortality was observed in all the treatments during the study period. Furthermore, no significant changes in organ weight were observed. Visually Group-III (Kalsiwin 1000 mg) shows a slight increase in left kidney weight [Tables 6 and 7].

Effect of Kalsiwin on Kidney X-ray Analysis

In the radiographic analysis, renal calculi were observed in Group-III, out of three males two males were affected with

Table 1: Effect of Kalsiwin on body weight changes

Treatment	Initial body weight	Final body weight	Changes in body weight
Group-I (Normal control)	263.33±29.40	300.00±31.62	36.67±4.94
Group-II (Kalsiwin-500 mg)	261.67±28.10	310.00±30.11	48.33±4.01
Group-III (Kalsiwin-1000 mg)	265.00±31.17	311.67±37.54	46.67±1.99

Values are expressed as mean±standard error of mean, n=6

Table 2: Effect of Kalsiwin on feed intake and water intake

Treatment	Feed intake (in g)		Water intake (in ml)	
	Initial intake	Final intake	Initial intake	Final intake
Group-I (Normal control)	82±0.62	85±1.50	137±2.98	125±7.43
Group-II (Kalsiwin-500 mg)	87±0.51	95±2.52	142±4.57	115±7.88
Group-III (Kalsiwin-1000 mg)	91±0.49	87±1. 20	136±5.77	157±8.36

Values are expressed as mean±standard error of mean, n=6

Table 3: Effect of Kalsiwin on behavior and observational parameters

S. No.	Parameters	Group-I				Group-II				Group-III			
		Day -1	Day-31	Day -61	Day-91	Day -1	Day-31	Day -61	Day-91	Day -1	Day-31	Day -61	Day-91
1	Fur and skin	N	N	N	N	N	N	N	N	N	N	N	N
2	Eyes (Exophthalmos, Lacrimation)	N	N	N	N	N	N	N	N	N	N	N	N
3	Salivation	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
4	Respiration	N	N	N	N	N	N	N	N	N	N	N	N
5	Urination	N	N	N	N	N	N	N	N	N	N	N	N
6	Feces consistency	N	N	N	N	N	N	N	N	N	N	Loose stools	Loose stools
7	Diarrhea	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
8	Sedation	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
9	Sleep	N	N	N	N	N	N	N	N	N	N	N	N
10	Loss of righting reflex	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
11	Writhing	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
12	Itching	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
13	Straub phenomenon	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
14	Piloerection	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
15	Motor activity	N	N	N	N	N	N	N	N	N	N	N	N
16	Grip strength	N	N	N	N	N	N	N	N	N	N	N	N
17	Convulsion/Tremor/Coma	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB

N: Normal, AB: Absent

Table 4: Effect of Kalsiwin on hematological parameters

Treatment	Group-I (Normal control)	Group-II (Kalsiwin-500 mg)	Group-III (Kalsiwin-1000 mg)
RBC (Million/cmm)	7.65±0.53	7.56±0.67	6.75±0.70
Hb (g/dL)	13.43±0.49	14.00±0.58	13.50±0.31
PLT (Lakhs/cmm)	7.31±0.34	4.09±0.90	4.99±1.53
HCT (%)	46.13±1.41	49.43±1.15	47.37±2.53
RDW (%)	13.60±0.32	13.23±0.57	13.27±0.55
MCV (Cu. Microns)	60.33±2.16	56.93±8.34	58.30±2.76
MCH (pg)	17.43±0.47	18.17±0.74	17.97±0.09
MCHC (g/dL)	28.93±0.33	28.50±0.85	29.40±0.56
WBC (cells/cumm)	4633.33±371.18	4400.00±311.49	4400.00±818.13
Neutrophils (%)	45.67±6.01	51.33±1.45	46.00±6.03
Lymphocytes (%)	39.67±11.57	39.67±2.33	45.67±6.77
Eosinophils (%)	4.33±0.36	3.00±0.58	1.67±0.33
Monocytes (%)	4.00±1.53	6.00±0.58	7.00±1.53

Values are expressed as mean±standard error of mean, n=6. Symbols represent statistical significance: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$

Table 5: Effect of Kalsiwin on biochemical parameters

Treatment	Group-I (Normal control)	Group-II (Kalsiwin-500 mg)	Group-III (Kalsiwin-1000 mg)
Serum glutamic oxaloacetic transaminase (mg/dL)	60.67±6.39	42.33±10.11	51.33±9.62
Serum glutamic pyruvic transaminase (mg/dL)	51.67±5.04	46.67±5.81	51.00±15.63
Alkaline phosphatase (mg/dL)	124.67±16.18	107.00±5.00	105.67±7.31
Gamma-glutamyl transferase (mg/dL)	12.33±0.67	13.33±0.67	16.33±1.33*
T. Bilirubin (mg/dL)	4.20±0.44	4.83±0.32	5.23±0.22
Albumin (g/dL)	5.73±0.38	5.73±0.69	4.03±0.24*
Globulin (g/dL)	3.77±0.29	3.43±0.24	2.73±0.15*
Total cholesterol (mg/dL)	95.00±3.06	96.33±1.20	97.67±0.67
Urea (mg/dL)	32.33±2.60	33.67±2.85	34.00±5.03
Creatinine (mg/dL)	0.6±0.12	0.73±0.18	0.33±0.03

Values are expressed as mean±standard error of mean, n=6. Symbols represent statistical significance: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$

Table 6: Effect of Kalsiwin on livability

Treatment	Dead/Total	Dead %	Survival %
Group-I (Normal control)	0/6	0	100
Group-II (Kalsiwin-500 mg)	0/6	0	100
Group-III (Kalsiwin-1000 mg)	0/6	0	100

renal calculi in Group-III. Group-II did not show any renal calculi occurrence [Figure 1].

Effect of Kalsiwin on Histopathological Analysis

Histopathological analysis of Group-I and II liver showed normal hepatocyte structure and hepatic sinusoidal lymphocytes. In Group-III observed apoptosis in the hepatocytes, marked lipofuscin deposition, spotty necrosis in numerous zones, increased Kupffer cell number, and marked vacuolar degeneration in the hepatocytes. In Group-I and II kidney showed histologically normal glomeruli with a normal density per unit area, normal tubules in all their segments, and a normal medulla having well-glycogenized nuclei. The interstitium is minimal in volume and normal. Group-III kidney showed glomerular hemorrhage, loss of mesangial cells, interstitial hemorrhagic necrosis in both the cortical and medullary zones, multiple zones of confluent tubular necrosis, numerous red cell casts, and diffuse lipofuscin deposition. Intestine showed a simple layered architecture with a simple mucosal fold pattern, short villi, and the muscularis propria was normal in Group-I. In Group II and III (Kalsiwin treated) showed a simple layered architecture with marked mucosal hyperplasia and long villi [Figure 2].

Table 7: Effect of Kalsiwin on relative organ weight

Treatment	Liver	Kidney		Heart	Spleen
		R	L		
Group-I (Normal control)	2.79±0.25	0.32±0.02	0.34±0.11	0.43±0.09	0.20±0.10
Group-II (Kalsiwin-500 mg)	3.02±0.09	0.31±0.01	0.33±0.06	0.40±0.11	0.22±0.03
Group-III (Kalsiwin-1000 mg)	3.16±0.14	0.36±0.02	0.42±0.12	0.48±0.15	0.26±0.01

Values are expressed as mean±standard error of mean, *n*=6. Symbols represent statistical significance: ***P<0.001, **P<0.01, *P<0.05

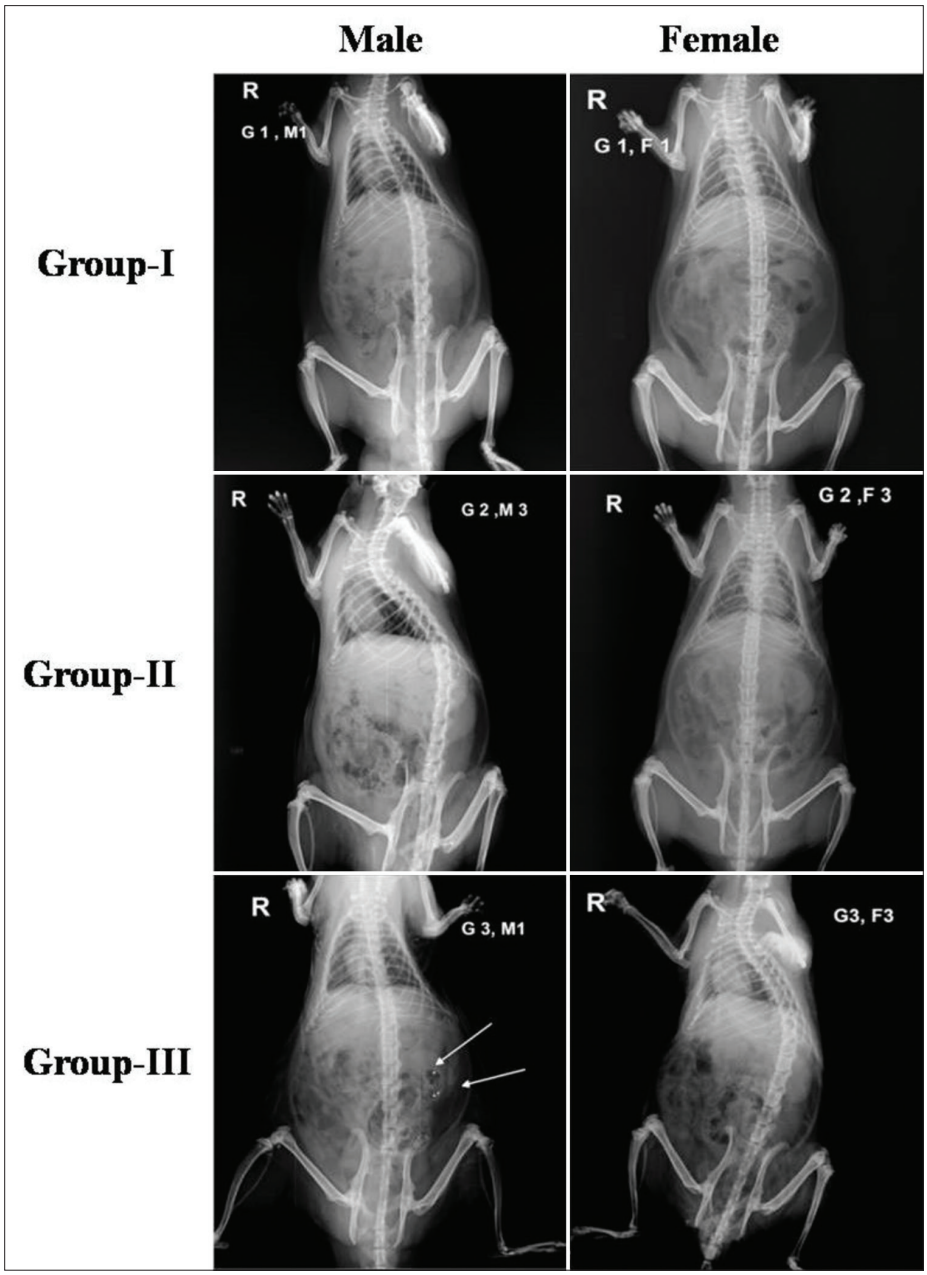

Figure 1: Effect of Kalsiwin on kidney X-ray analysis

Sub-chronic Effect of Kalsiwin on Ulcer index

Figure 3 illustrate the sub-chronic effect of Kalsiwin on GI smooth muscles. Group-III rat stomach showed severe gastric lesions and ulcer index. Group-I and II showed normal GI mucosa.

DISCUSSION

Nowadays herbal medicines are extensively using in alternative or complementary therapies due to their abundant therapeutic application and efficacy. However, many effective herbal medicines are available, and some have been

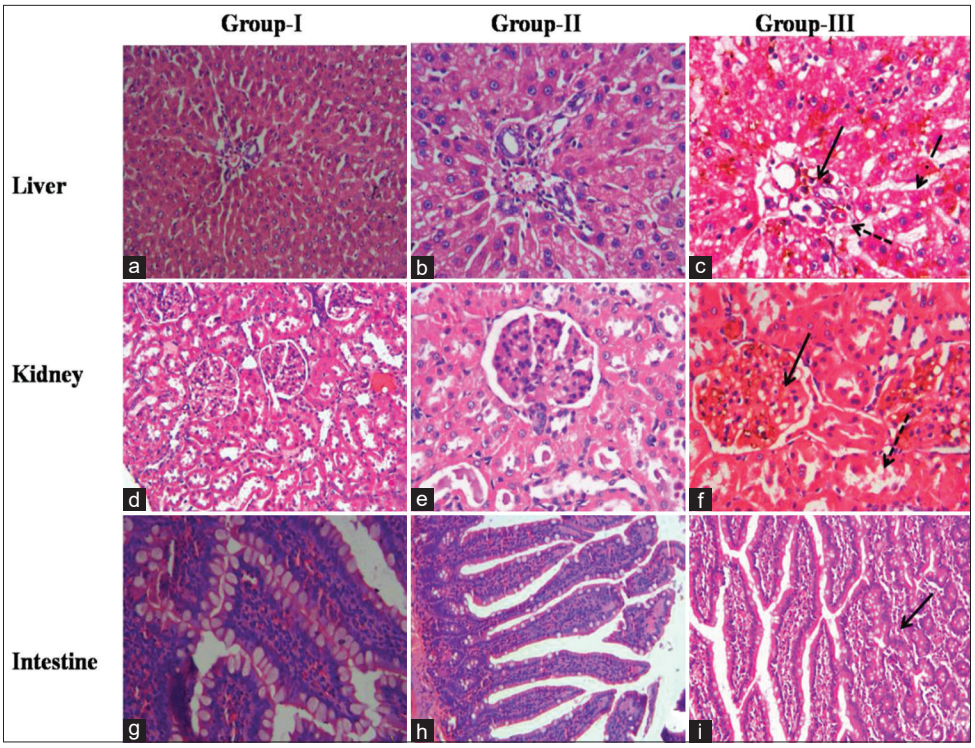

Figure 2: Sub-chronic effect of Kalsiwin on H and E staining histopathology of liver, kidney, and intestine. (a, b, and c) indicates the histological changes in liver, in figure (a and b) shows normal hepatocyte structure and hepatic sinusoidal lymphocytes, in figure (c) there was spotty necrosis in numerous zones, increased Kupffer cells and marked vascular degeneration. (d, e, and f) indicates the histological changes in kidney; in figure (d and e) shows histologically normal glomeruli, tubules, and a normal medulla having well-glycogenized nuclei. (f) shows glomerular hemorrhage, loss of mesangial cells, interstitial hemorrhagic necrosis, and diffuses lipofuscin deposition. (g, h and i) indicates the histological changes in intestine, in figure (g and h) shows a simple layered architecture with a simple mucosal fold pattern, short villi, and normal muscularis propria, in figure (i) shows a simple layered architecture with marked mucosal hyperplasia and long villi

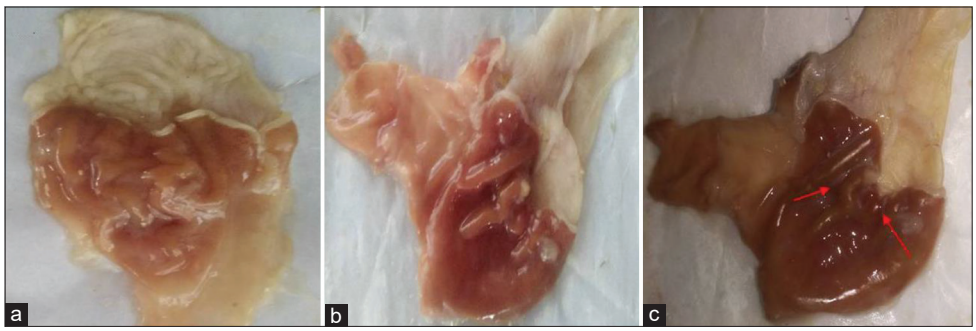

Figure 3: Sub-chronic effect of Kalsiwin on ulcer index. (a) Shows normal GI mucosa of normal control, (b) shows normal GI mucosa of Kalsiwin 500 mg (therapeutic dose level) treated rat, (c) shows ulcerated mucosa with vascular congestions in Kalsiwin 1000 mg (high dose level) treated rats

verified by clinical trials, but still, safety is questionable by practitioners as well as consumers.[9,10] Kalsiwin is one of the herbal calcium supplement supplied by SKM Siddha and Ayurveda, India, it has already demonstrated effectiveness in clinical practice, and its toxicity has not been fully explored. In this study, we performed sub-chronic toxicity studies in Wistar rats to evaluate its safety index on chronic use.

The therapeutic dose of Kalsiwin is 500 mg/day for adults; the same dose level 45 mg/kg for rats was used to investigate the NOAEL. Kalsiwin 1000 mg/day adult dose, 90 mg/kg for rats which is double the therapeutic dose used to detect the MTD in rats.

In the sub-chronic toxicity study, no significant changes were observed in the changes in body weight, feed, and water intake in all the treatments. The chronic administration of Kalsiwin also does not produce any observational and behavioral changes (including breathing, movement, and twitch). In addition, no mortality was observed throughout

the study period. Furthermore, no significant change in relative organ weights in the 90 days (Sub-chronic) Kalsiwin administered rats. On 61st and 91st-day, Kalsiwin 90 mg/kg administered animals produce loose stools. Chronic calcium supplements may increase the incidence of constipation, severe diarrhea, and abdominal pain.[11] The results of this study also suggested that chronic use of Kalsiwin at the dose higher than the recommended clinical dose leads to loose stools. Hence, Kalsiwin is nontoxic at the administered dose up to 45 mg/kg in rats.

The analyses of hematological parameters are the perceptive indicators of the clinical toxicity of drugs and chemical molecules.[12-14] Repeated dosing of Kalsiwin over 90 days does not produce any significant changes in the erythrocytes, Hb, platelets, and differential leukocyte counts when compared with normal control. Hence, it reveals that the chronic administration of Kalsiwin does not alter the hematological parameters.

Serum hepatic enzymes SGOT, SGPT, ALP, GGT, bilirubin, albumin, and globulin are the hallmark indicator of liver function. The exposure of toxicants to hepatocytes, damage to its membrane may lead to leakage and serum elevation of these enzymes.[15,16] Sub-chronic administration of Kalsiwin showed no significant difference in the serum SGOT, SGPT, ALP, and bilirubin levels. However, the level of GGT, serum albumin, and globulin were showed mild significant alteration in Kalsiwin higher clinical dose level. GGT is an enzyme that is found in many organs throughout the body, with the highest concentrations found in the liver. GGT is elevated in the blood circulation during the damage of the liver or bile ducts.[17] In this study, Kalsiwin 1000 mg treatment group indicates the significant increases in GGT. The elevated total cholesterol, lower serum albumin, and globulin level indicate liver disease or an inflammatory disease.[18,19] Results of this study also showed Kalsiwin 90 mg/kg treatment group indicates the significant decreases in serum albumin and globulin levels. It all indicates the abnormality of liver function in the sub-chronic administration of Kalsiwin above the clinical dose level. Results of histopathological changes also suggested that the hepatotoxic nature of Kalsiwin attributed to chronic use.

Serum urea and creatinine are critical biochemical markers to determine the kidney function and toxicity of drugs and chemicals.[20,21] There was no significant change in serum urea and creatinine level changes on the sub-chronic administration of Kalsiwin. Besides, the X-ray analysis results of treated rat kidney showed developed renal calculi in two out of three males in the higher clinical dose level of Kalsiwin on chronic use. Histopathological results of the kidney also illustrated that Kalsiwin 1000 mg dose level produced tubular calcification and necrosis. It might indicate that chronic uses of Kalsiwin at a higher dose level may lead to nephrotoxicity.

Evaluation of the ulcerogenic property of herbal formulation is one of the key parameters for evaluating the safety index of a drug.[22] The presence of ulceration in the stomach mucosa of the rats treated with Kalsiwin 90 mg/kg/day for 90 days indicates the ulcerogenic nature of Kalsiwin above its therapeutic dose. Kalsiwin at the therapeutic dose level does not show any ulceration on sub-chronic use.

CONCLUSION

Sub-chronic 90 days oral repeated dosing studies of Kalsiwin in rats reveal that NOAEL of Kalsiwin is 45 mg/kg, which is equivalent to the human therapeutic dose of 500 mg/day. The MTD of Kalsiwin is <90 mg/kg in Wistar rats, which is equivalent to the human therapeutic dose of 1000 mg/day. Therefore, results of this study suggest that oral chronic administration of Kalsiwin at the human therapeutic dose of 500 mg/day is considered to be safer.

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