

Oral Acute and Subacute Toxicity Studies with Kalpaamruthaa, a Modified Indigenous Preparation, on Rats

Rajendran Mythilypriya,^a Palanivelu Shanthi,^b and Panchanatham Sachdanandam^{*,a}

^aDepartment of Medical Biochemistry, Dr. ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai–600 113, India and ^bDepartment of Pathology, Dr. ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai–600 113, India

(Received September 4, 2006; Accepted April 22, 2007)

Kalpaamruthaa (KA), a modified indigenous Siddha formulation constitutes *Semecarpus anacardium* nut milk extract, *Emblica officinalis* and honey. KA is evaluated for its behavioral and toxicological effects and also its consequence on biochemical and histological variations. Acute and subacute toxicity studies with KA were done on Wistar Albino rats. During acute toxicity study (72 hr), there were no any adverse effects found in the general behavior and mortality at any dose level given (50–2000 mg/kg b.wt.). In subacute toxicity study (30 days) KA (50, 100, 250 and 500 mg/kg b.wt.) did not cause any changes in hematological and biochemical parameters with the exception of a transient rise in hemoglobin, leukocyte count, free fatty acid, plasma and urine creatinine and a significant decrease in blood glucose, total cholesterol, triglyceride and phospholipid levels. The changes observed are significant only at the highest dosage of 500 mg/kg b.wt. Further, histopathological examination of vital organs showed normal architecture suggesting no morphological disturbances; it can be considered that KA is safe and non toxic.

Key words — Kalpaamruthaa, acute toxicity study, 30 day's subacute toxicity study, wistar albino rats

INTRODUCTION

Traditional medicines are used by about 60% of the World's population. These are not only used for primary health care, not just in rural areas of developing countries, but also in developed countries, where modern medicines are predominantly used.¹⁾ In Indian systems of medicine, most practitioners formulate and dispense their own recipes. Hence, this requires proper research and its documentation. Kalpaamruthaa (KA), an indigenous modified Siddha formulation consists of *Semecarpus anacardium* nut milk extract (SA) and *Emblica officinalis* fruit. In order to promote intellect and prevent senility and for longevity, honey was also added to this preparation. Both *Semecarpus anacardium* and *Emblica officinalis* independently are proved to exhibit a vital role in traditional and mod-

ern medicines, because of their wider pharmacological activities.

SA has been reported to have antioxidant, membrane stabilizing, immunomodulatory,²⁾ anticancerous^{3,4)} and antiarthritic⁵⁾ activities. The anti-inflammatory effects of *Semecarpus anacardium* nut on acute inflammations of both immunological and non-immunological origin have also been demonstrated.⁶⁾ It also possesses a promising hypolipidemic⁷⁾ and antiatherosclerotic⁸⁾ activities.

Emblica officinalis commonly known as Amla, a rich source of vitamin C have been used in Ayurveda as a potent rasayana. The rasayanas are used to promote health and longevity by increasing defence against disease, arresting the aging process and revitalizing the body's debilitated conditions.⁹⁾ *Emblica officinalis* has been reported to have antioxidant,¹⁰⁾ hypolipidemic,¹¹⁾ hypoglycemic,¹²⁾ adaptogenic,¹³⁾ hepatoprotective,¹⁴⁾ anti-tumour,¹⁵⁾ cytoprotective and immunomodulating¹⁶⁾ properties.

A wealth of literature is available on the occurrence and health benefits of vitamin C.¹⁷⁾ Further, there were several evidences attributing the biolog-

*To whom correspondence should be addressed: Department of Medical Biochemistry, Dr. ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai–600 113, India. Tel.: +91-44-24925548; Fax: +91-44-24926709; E-mail: psachdanandam2000@yahoo.co.in

ical and therapeutic effects of amla fruits to their rich vitamin C content.¹⁸⁾ While the bioactivity and pharmacokinetics of ascorbic acid is well known, that of the flavonoids is very much under debate.¹⁹⁾ Numerous studies have demonstrated the protective properties of the polyphenolic flavonoids. Interactions between flavonoids and ascorbic acid have been documented. Ascorbate is reported to have flavonoid protective²⁰⁾ and flavonoid enhancing activities.²¹⁾ Also, stabilization of vitamin C at sub-optimal concentrations by flavonoids is well established.²²⁾ Interestingly, it was reported that the vitamin C like activity of Amla was due to the presence of hydrolysable tannins such as emblicanin A, emblicanin B, puningluconin, pedunculagin and galloelagitanins.¹⁰⁾

Hence, in order to improve the bioactivity of SA, which has been reported for the presence of carbohydrate, phenols and flavonoids²³⁾ the combination with *Emblica officinalis* was tried with a view to promote the curative potency. On preliminary phytochemical analysis, KA gave positive results for polyphenols (flavonoids and tannins), triterpenoids, steroids, sugars and proteins. It also contains other components such as minerals (Fe, Ca, Mg, P, Na, K and Zn), vitamins (ascorbic acid, thiamine, riboflavin, niacin, pyridoxine, cyanocobalamine and folic acid) and fiber.

A plant to be identified for traditional medicine, a systemic approach through experimental and clinical validation of efficacy is required, as is done in modern medicine; animal toxicity studies are also required to establish the potential adverse effects of KA. Though SA has been proved to be non-toxic upto the dose level of 2000 mg/kg b.wt.,²⁴⁾ the aim of the present study was to carry out basic toxic evaluation and to establish the safety of SA in combination with *Emblica officinalis*. This article focus on acute and subacute toxicity evaluation of KA in Wistar Albino rats.

MATERIALS AND METHODS

Drug and Chemicals — SA has been prepared according to formulary of Siddha medicine.²⁵⁾ To this, a fresh powder of *Emblica officinalis* fruit and honey was added. All other chemicals and solvents used were of analytical grade.

Acute Toxicity Study — Healthy Wistar albino rats of either sex weighing 100 ± 20 g were divided into 6 Groups of 6 animals each. The animals were

housed under standard conditions and room temperature ($25 \pm 2^\circ\text{C}$) was controlled. All animals were fed with standard rat pelleted diet (M/S Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and had free access to tap water *ad libitum*. The study has got the approval from the Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). KA was administered orally through gastric intubation in olive oil at doses of 50, 100, 250, 500, 1000, and 2000 mg/kg b.wt. and control group received 0.5 ml of the vehicle alone. The animals were observed continuously for 72 hr for any signs of behavioral changes, toxicity and mortality.

Subacute-acute Toxicity Study — Wistar albino rats of either sex weighing 170 ± 10 g were divided into 5 Groups [Groups I–V] of 6 animals each and were housed under the same conditions as described above. KA was administered for 30 days at doses of 50, 100, 250, and 500 mg/kg b.wt. respectively. The control animals (Group I) received 0.5 ml of the vehicle alone. Toxic manifestations and mortality were monitored daily and b.wt. changes were recorded every 7 days till the end of the study.

Clinical Test Parameters — At 28th day, 24 hr urine samples were collected by placing the animals in the metabolic cage with free access to tap water. Toluene is used as a preservative while collecting the sample. The collected urine was used for biochemical estimations. At 30th day, animals were fasted for 12 hr, then anesthetized with ether and blood was collected from Jugular vein in two tubes: one with EDTA for immediate analysis of hematological parameters and to separate plasma for biochemical estimations, the other without any additives and was centrifuged at 4000 rpm at 4°C for 10 min to obtain the serum. Both the plasma and serum were stored at -20°C until analyzed for biochemical parameters. Animals were then sacrificed by ether anesthesia and liver, kidney, lung, heart, spleen and adrenals were dissected out, washed and transferred to an ice-cold saline solution. The organs were weighed and portions of these organs were fixed in 10% formalin for histopathological examinations. Ten percent tissue homogenates of liver and kidney were prepared by homogenizing a weighed amount of tissues in 0.1 M tris HCl buffer, pH 7.4. The homogenates were analyzed for biochemical parameters, alkaline phosphatase (ALP),²⁶⁾ glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transami-

nase (GPT).²⁷⁾ Further, lipids were extracted from the tissues by the method of Folch *et al.*²⁸⁾ and the lipid profile: total cholesterol (TC),²⁹⁾ triglyceride (TG),^{30,31)} phospholipids (PL)³²⁾ and free fatty acids (FFA)³³⁾ were also determined. The hematological parameters like Hemoglobin, Red Blood Cell (RBC), White Blood Cell (WBC) and Packed Cell Volume (PCV)³⁴⁾ were determined by usual standardized laboratory method. The biochemical parameters, blood glucose,³⁵⁾ urea,³⁶⁾ plasma protein,³⁷⁾ uric acid,³⁸⁾ creatinine,³⁹⁾ TC,²⁹⁾ TG,^{30,31)} PL³²⁾ and FFA³³⁾ were determined. Serum ALP,²⁶⁾ GOT²⁷⁾ and GPT²⁷⁾ were also determined.

Urine Analysis—In 24 hr urine sample, protein,³⁷⁾ uric acid³⁸⁾ and creatinine³⁹⁾ were determined.

Statistical Analysis—The values are expressed as mean \pm standard deviation (S.D.). Results were analyzed statistically using student's *t*-test. The significant difference between the groups are considered at $p < 0.05$ level.

RESULTS

Acute Toxicity Study

There was no mortality or any signs of behavioral changes or toxicity observed after oral administration of KA upto the dose level of 2000 mg/kg b.wt. in rats.

Subacute Toxicity Study

Effect of KA on Body and Organ Weights

The body (at 28th day) and organ weight changes of control and KA treated rats are presented in Table 1. There were no significant differences in the body weight and organ weight gain between the control and KA treated groups. Moreover, no lethality was recorded for any dose upto the maximum of 500 mg/kg during the 30 days period of treatment.

Effect of KA on the Hematological and Biochemical Parameters

The effect of KA on the hematological parameters is presented in Table 2. The hemoglobin and leucocytes count were found to be significantly in-

Table 1. Effect of KA on Organ Weight Changes in Control and Treated Rats of Subacute Toxicity Studies

Organ weight	Group I (Control)	Group II (50 mg/kg b.wt.)	Group III (100 mg/kg b.wt.)	Group IV (250 mg/kg b.wt.)	Group V (500 mg/kg b.wt.)
Liver (g/100 g)	4.51 \pm 0.44	4.03 \pm 0.39	4.50 \pm 0.41	4.86 \pm 0.47	4.51 \pm 0.44
Kidney (g/100 g)	1.05 \pm 0.10	1.18 \pm 0.13	1.1 \pm 0.11	1.03 \pm 0.10	1.07 \pm 0.12
Lung (g/100 g)	0.83 \pm 0.20	0.93 \pm 0.09	0.90 \pm 0.08	0.92 \pm 0.10	0.92 \pm 0.11
Heart (g/100 g)	0.58 \pm 0.09	0.60 \pm 0.03	0.59 \pm 0.09	0.59 \pm 0.07	0.60 \pm 0.08
Spleen (g/100 g)	0.64 \pm 0.07	0.58 \pm 0.07	0.57 \pm 0.06	0.56 \pm 0.06	0.57 \pm 0.06
Adrenal (mg/100 g)	40 \pm 5.14	42.1 \pm 6.03	42.89 \pm 4.58	44.11 \pm 6.66	43.98 \pm 5.21
Mortality (%)	Nil	Nil	Nil	Nil	Nil
Body weight (28 th day)	218.5 \pm 20.5	216.0 \pm 22.5	215.5 \pm 19.0	217.0 \pm 22.5	212.5 \pm 20.0

Values are expressed as mean \pm S.D. for 6 rats. Comparisons were made between Group I with Groups II, III, IV and V.

Table 2. Effect of KA on Haematological and Biochemical Parameters in Treated and Control Rats of Subacute Toxicity Studies

Parameters	Group I (Control)	Group II (50 mg/kg b.wt.)	Group III (100 mg/kg b.wt.)	Group IV (250 mg/kg b.wt.)	Group V (500 mg/kg b.wt.)
Protein (g/dl)	7.1 \pm 0.6	6.33 \pm 0.65	6.53 \pm 0.66	7.66 \pm 0.79	7.80 \pm 0.71
Urea (mg/dl)	15.9 \pm 1.44	15.81 \pm 2.49	16.04 \pm 2.43	16.14 \pm 2.32	16.24 \pm 2.57
Uric acid (mg/dl)	1.36 \pm 0.22	1.22 \pm 0.20	1.27 \pm 0.14	1.43 \pm 0.16	1.50 \pm 0.25
Creatinine (mg/dl)	0.60 \pm 0.09	0.65 \pm 0.11	0.68 \pm 0.06	0.70 \pm 0.08	0.84 \pm 0.13*
Glucose (mg/dl)	79.27 \pm 5.79	78.37 \pm 8.12	76.27 \pm 7.89	71.56 \pm 6.26	72.15 \pm 4.96*
Hb (g/dl)	14.61 \pm 1.24	14.98 \pm 2.12	15.64 \pm 1.85	16.37 \pm 1.62	16.99 \pm 2.24*
RBC (millions/mm ³)	4.64 \pm 0.48	4.28 \pm 0.59	4.73 \pm 0.61	4.45 \pm 0.47	4.79 \pm 0.53
WBC (thousands/mm ³)	6.32 \pm 0.58	6.83 \pm 0.75	7.01 \pm 0.68	7.10 \pm 0.69	7.22 \pm 0.72*
PCV (%)	44.16 \pm 5.47	41.00 \pm 4.54	38.56 \pm 4.24	42.33 \pm 4.75	44.83 \pm 5.16

Values are expressed as mean \pm S.D. for 6 rats. Comparisons were made between Group I with Groups II, III, IV and V. The symbol * also represent the statistical significance at $p < 0.05$.

creased ($p < 0.05$) in Group V KA treated animals when compared to the control rats. All the other parameters (RBC and PCV) in all treated groups remained within the normal limits.

Table 2 depicts the plasma protein, uric acid, creatinine, blood glucose and urea respectively. A significant decrease ($p < 0.05$) in blood glucose level was observed in Group V KA treated rats when compared to control group. Plasma protein, uric acid and blood urea of both control and treated groups were remained normal without any significant difference, whereas a significant increase in

creatinine ($p < 0.05$) was found at the highest dosage of KA treated rats.

Table 3 represents the marker enzymes (GOT, GPT and ALP) in serum, liver and kidney. There were no significant changes observed in GOT, GPT and ALP levels in serum, liver and kidney.

Effect of KA on Lipid Profiles of Control and KA Treated Groups

Table 4 shows the plasma, liver and kidney lipid profiles (TC, TG, PL and FFA). Except a significant increase and decrease ($p < 0.05$) in the lev-

Table 3. Effect of KA on Marker Enzymes in Serum, Liver and Kidney in Control and Treated Rats

Parameters	Group I (Control)	Group II (50 mg/kg b.wt.)	Group III (100 mg/kg b.wt.)	Group IV (250 mg/kg b.wt.)	Group V (500 mg/kg b.wt.)
Serum (units)					
GOT	38.58 ± 3.92	39.25 ± 3.21	40.01 ± 6.38	37.11 ± 5.07	37.81 ± 4.88
GPT	24.42 ± 3.56	27.35 ± 3.92	25.37 ± 3.13	25.44 ± 2.64	25.96 ± 2.01
ALP	0.017 ± 0.01	0.011 ± 0.068	0.048 ± 0.067	0.013 ± 0.016	0.014 ± 0.011
Liver (units)					
GOT	34.86 ± 3.29	34.99 ± 5.62	35.02 ± 4.99	35.37 ± 4.27	36.23 ± 3.71
GPT	98.64 ± 10.05	99.22 ± 9.37	97.28 ± 8.91	99.65 ± 11.35	99.88 ± 10.67
ALP	0.026 ± 0.004	0.024 ± 0.004	0.027 ± 0.005	0.028 ± 0.004	0.026 ± 0.003
Kidney (units)					
GOT	23.66 ± 2.81	25.78 ± 4.64	22.95 ± 3.38	24.38 ± 3.30	23.89 ± 4.54
GPT	30.12 ± 4.69	29.35 ± 3.22	32.37 ± 4.85	30.83 ± 2.88	30.57 ± 4.9
ALP	0.162 ± 0.002	0.169 ± 0.024	0.155 ± 0.011	0.152 ± 0.016	0.164 ± 0.009

Values are expressed as mean ± S.D. for 6 rats. Units: GOT and GPT; μ moles of pyruvate liberated/min/mg protein, ALP; μ moles of phenol liberated/min/mg protein. Comparisons were made between Group I with Groups II, III, IV and V.

Table 4. Effect of KA on Lipid Profile in Treated and Control Rats

Parameters	Group I (Control)	Group II (50 mg/kg b.wt.)	Group III (100 mg/kg b.wt.)	Group IV (250 mg/kg b.wt.)	Group V (500 mg/kg b.wt.)
Plasma (mg/dl)					
TC	50.18 ± 5.01	49.14 ± 6.27	46.06 ± 5.64	45.58 ± 5.09	39.75 ± 3.88*
TG	120.36 ± 13.97	127.77 ± 15.31	131.43 ± 12.69	137.88 ± 14.05	161.06 ± 15.6*
PL	110.41 ± 11.82	113.74 ± 8.94	117.22 ± 10.52	109.82 ± 12.08	93.91 ± 10.96*
FFA	2.16 ± 0.32	2.08 ± 0.37	2.20 ± 0.18	2.39 ± 0.39	2.84 ± 0.29*
Liver (mg/g wet tissue)					
TC	8.60 ± 0.99	8.76 ± 0.91	8.61 ± 1.05	8.92 ± 1.08	7.41 ± 0.85*
TG	15.01 ± 2.25	14.38 ± 1.81	14.56 ± 1.82	14.42 ± 1.6	12.07 ± 1.84*
PL	21.65 ± 2.37	23.85 ± 3.07	24.77 ± 2.52	24.91 ± 3.01	18.16 ± 2.27*
FFA	3.20 ± 0.31	3.14 ± 0.38	3.21 ± 0.37	3.32 ± 0.28	3.80 ± 0.41*
Kidney (mg/g wet tissue)					
TC	4.71 ± 0.49	4.86 ± 0.69	4.97 ± 0.58	4.91 ± 0.52	3.92 ± 0.41*
TG	6.10 ± 0.58	6.21 ± 0.83	6.27 ± 0.71	6.18 ± 0.64	5.27 ± 0.55*
PL	13.74 ± 2.13	14.68 ± 1.42	13.99 ± 1.07	15.41 ± 2.1	11.33 ± 0.97*
FFA	3.57 ± 0.29	3.44 ± 0.36	3.64 ± 0.31	3.80 ± 0.41	4.10 ± 0.28*

Values are expressed as mean ± S.D. for 6 rats. Comparisons were made between Group I with Groups II, III, IV and V. The symbol * also represent the statistical significance at $p < 0.05$.

Table 5. Effect of KA on Biochemical Parameters in Urine in Treated and Control Rats

Parameters	Group I (Control)	Group II (50 mg/kg b.wt.)	Group III (100 mg/kg b.wt.)	Group IV (250 mg/kg b.wt.)	Group V (500 mg/kg b.wt.)
Protein (g/dl)	6.81 ± 0.46	6.61 ± 0.42	6.45 ± 0.59	6.95 ± 0.64	6.90 ± 0.50
Urea (mg/dl)	2.04 ± 0.25	2.11 ± 0.17	2.21 ± 0.23	2.19 ± 0.24	2.24 ± 0.18
Uric acid (mg/dl)	0.091 ± 0.011	0.088 ± 0.019	0.09 ± 0.007	0.092 ± 0.011	0.095 ± 0.009
Creatinine (mg/dl)	0.62 ± 0.065	0.64 ± 0.12	0.68 ± 0.10	0.66 ± 0.09	0.74 ± 0.05*

Values are expressed as mean ± S.D. for 6 rats. Comparisons were made between Group I with Groups II, III, IV and V. The symbol * also represent the statistical significance at $p < 0.05$

els of plasma TG and TC, PL respectively, there were a significant decrease ($p < 0.05$) in the levels of TC, PL and TG in liver and kidney at a dose of 500 mg/kg b.wt. of KA. In contrast a significant increase ($p < 0.05$) were observed in the level of plasma, liver and kidney FFA in Group V of KA treated groups when compared to control rats.

Effect of KA on urine parameters

Table 5 portrays the urine levels of protein, urea, uric acid and creatinine. Creatinine level in urine was observed to be significantly increased ($p < 0.05$) only at 500 mg/kg b.wt. of KA. All the other parameters were found to be non significantly varying when compared with that of the control rats.

DISCUSSION

Herbal medicines have received greater attention as an alternative to clinical therapy and the demand for these remedies has currently increased. Experimental screening method is important in order to ascertain the safety and efficacy of traditional and herbal products and also to establish the active component of the herbal products.⁴⁰⁾ In acute toxicity study, there was no any mortality observed upto the maximum dose level of 2000 mg/kg b.wt. of the KA administered orally, which the single high dose is recommended by OECD guidelines-423⁴¹⁾ for testing acute toxicity. Thus, our test suggested that KA does not cause any apparent acute toxicity. The changes in body weight have been used as an indicator of adverse effects of KA and chemicals.⁴²⁾ Since there were no changes in animal behavior, body and organ weights at all doses of the treated rats when compared to the control group, the present results suggest that at the oral doses administered, the KA is non-toxic.

With the exception of a transient increase in hemoglobin and WBC count there were no significant alterations in the hematological parameters.

This increase in the hemoglobin level might be due to the increased absorption of iron by vitamin C in KA and the increase in the WBC level may indicate the impact of KA in boosting the immune system of treated groups. The increase might be speculated due to the immunopotentiating effect of KA.

The significant decrease ($p < 0.05$) in blood glucose level in Group V KA treated rats might be due to the presence of hypoglycemic components in KA. This again emphasizes the advantageous effect of KA on to hyperglycemic subjects. Raised urea and non-protein level in blood have been observed with impaired renal function (or) in acute renal failure.⁴³⁾ In the present study, a significant increase ($p < 0.05$) in both plasma and urine creatinine level at the highest dosage of KA treated group indirectly manifests the non-hazardous effect of KA in maintaining the homeostasis of protein and non-protein nitrogen in the body fluids of treated and control rats. These findings thereby illustrate the nontoxic effect of KA on renal function in treated rats when compared to control groups.

Transaminases (GPT and GOT) and ALPs are good indices of liver and kidney damage respectively.⁴⁴⁾ There were no deleterious changes found in the level of transaminases and ALPs in serum, liver and kidney of treated groups when compared with the control rats. Hence, from the above outcomes it can be delineated that KA did not provoke any detrimental effects on liver and kidney tissues in treated groups. The organ protective efficacy of KA is further confirmed by the histological assessment.

The decrease in plasma TC and PL levels might be due to the presence of hypolipidemic agents in KA. Earlier, it was reported that ascorbic acid increases cholesterol transformation to its degradation product bile acids, through stimulation of the enzyme 7α -hydroxylase which catalyses the first step in the conversion of cholesterol to 7α -hydroxycholesterol.⁴⁵⁾ Bile acid adsorption by dietary fiber *in vitro* has also been reported for many fiber types.⁴⁶⁾ In addition polyphenols such as

flavonoids and tannins have been shown to have numerous health protective benefits of which include lowering of blood lipids. Furthermore recent reports have suggested that several plant sterols reduce serum cholesterol by the inhibition of intestinal cholesterol absorption.⁴⁷⁾ Thus, it can be suggested that the synergistic interaction of flavonoids, tannins, sterols, vitamin C and fiber contents in KA may impart hypolipidemic property to KA.

One of the reasons for the increase in FFA levels in plasma, liver and kidney might be due to the hypoglycemic effect of KA, wherein the drug may stimulate total glucose utilization and/or decrease glucose mobilization from liver; and stimulate the efflux of FFA from adipose tissue,^{48,49)} thereby decreasing the blood glucose level and increasing the plasma and liver FFA concentration (or) KA might have direct effect on adipose tissue in mobilization of FFA. The fate of this FFA in liver might be either utilized for TG synthesis resulting in the increased level of plasma TG in Group V rats as evidenced in the present study or the FFA might oxidized in order to revitalize the energy (because of decrease in blood glucose). Though the changes in the level of plasma cholesterol being an indirect indicator of liver function,⁵⁰⁾ the organ protective efficacy of KA is further confirmed by the histological assessment.

Histopathological examination of selected organs (heart, liver, kidney, spleen and adrenals) from both treated and control animals showed normal architecture, suggesting no detrimental changes and morphological disturbances caused due to the administration of KA for 30 days.

In normal humans, the KA may not pose a danger to hypoglycemia, and hypolipidemia. So, in order to determine the long time efficacy of KA as a hypoglycemic and hypolipidemic agent, additional studies have to be performed in hyperglycemic and hyperlipidemic rats. Further, the increase in hemoglobin level and WBC count emphasize the beneficial effect of KA as a supplement.

In conclusion, KA can be considered safe, as it did not cause either any lethality or adverse changes with general behaviour of rats in acute toxicity study up to the dose of 2000 mg/kg b.wt. and also there were no observable detrimental effects caused by KA (up to 500 mg/kg b.wt.) in subacute toxicity study in rat model. Further, the above results substantiate the beneficial and enhanced pharmacological effects of the formulation, KA when compared with the independent activity of SA.²⁴⁾

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