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Toxic Effects of Oral Administration of Extracts of Dried Calyx of *Hibiscus sabdariffa* Linn. (Malvaceae)

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The effects of a 90-day oral administration of water and alcohol extracts of dried calyx of *Hibiscus sabdariffa* were evaluated in albino rats. Haematological, biochemical and histopathological changes were monitored every 30 days.

The death of the animals was preceded by a severe loss in weight, accompanied with diarrhoea in animals on the 2000 mg/kg dose. There was an increase in food intake (g) per kg body weight per day in the aqueous (A) and ethanol (E) 300 mg/kg extract groups. Significant reductions in the erythrocyte count with no difference in total leucocyte count were observed. The activity of aspartate aminotransferase (AST) was enhanced by the administration of aqueous and 50% ethanol extract with a significant increase in its level at higher doses ($p < 0.05$). Alanine aminotransferase (ALT) and creatinine levels were significantly affected by all the extracts at the different dose levels. However, aqueous extracts exhibited a significant increase in creatinine levels ($p < 0.05$) at higher doses. The cholesterol levels were generally not significantly affected by the extracts. No significant histopathological changes were observed, although there was a significant reduction in the weight of the spleen of the animals administered with ethanol and water extracts when compared with the control ($p < 0.01$). Other organs were of the same relative weight. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: chronic toxicity profile; *Hibiscus sabdariffa* extracts; rats.

INTRODUCTION

Hibiscus sabdariffa Linn (family Malvaceae) is an annual herb used for its medicinal properties in countries such as Thailand, Mali, China and Mexico. The calyces, which are rich in phenolic compounds contain gossypetin, glucoside, hibiscin, hibiscus anthocyanin and hibiscus protocatechuic acid, possess diuretic and choleretic effects, decreasing the viscosity of the blood, reducing blood pressure and stimulating intestinal peristalsis (Ali and Salih, 1991; Owulade *et al.*, 2004).

The pigments contained in flowers of the Hibiscus species have been identified as anthocyanins, namely cyanidin-3-glucoside and delphinidine-3-glucoside (Du and Francis, 1973; Nakamura *et al.*, 1990) which have found importance in food manufacture. Studies have shown that the crude extracts, and some of the constitutents, particularly the anthocyanins and protocatechuic acid, possess strong antioxidant activities *in vitro* and *in vivo* (Tanaka *et al.*, 1994; Tanaka *et al.*, 1995; Tsuda *et al.*, 1996; Tseng *et al.*, 1997; Wang *et al.*, 2000). The water infusions prepared from the dried calyx are taken as beverages in Nigeria, Thailand, Sudan, Mexico and some Arab countries, and also used for liver disorders (Chen *et al.*, 2003). The antihypertensive activity of water beverages made from the dried calyx of the plant has been established in animal models and human (Odigie *et al.*, 2003; Herrera-Arrelano *et al.*, 2004).

Acute and sub-acute toxicity profiles of different extracts of parts of this plant have been reported widely in the literature (Onyenekwe *et al.*, 1999; Akindahunsi and Olaleye, 2003; Orisakwe *et al.*, 2004; Ali *et al.*, 2005) with conflicting reports. Even though beverages prepared from this plant are widely taken in certain parts of the world, there is no literature on the chronic toxicity of extracts of the plant. The aim of this study therefore, was to evaluate the effect of a 90-day oral administration of the extracts of the dried calyx of *Hibiscus sabdariffa* in an animal model.

METHODOLOGY

Plant material. The dried flowers of *Hibiscus sabdariffa* were bought from Bodija Market, in Ibadan and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan. The dried calyces were further dried at 40 °C until a constant weight was obtained and pulverized to obtain a coarsely powdered material.

Extraction. All solvents used were all of analytical grade. One liter each of distilled water, 50% ethanol (water/ethanol, 50:50) and 100% ethanol were used to infuse 100 g each of the powdered plant material for 4 h. The extract obtained was decanted and the material was

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re-extracted with another 1 L of the same solvent. The extracts obtained for each solvent was pooled, filtered and dried *in vacuo* except for the water extract which could not be dried but was concentrated further. The amount of extract in the water extract was determined by weighing an equal volume of water.

Anthocyanin determination. The anthocyanin content of the dried calyx was determined by calculating the cyanindin-3-glucoside using a colorimetric method based on the ability of anthocyanins to produce a color at pH 1.0 that disappears at pH 4.5. This characteristic is produced by a pH dependent structural transformation of the chromophore. The colored oxonium ion predominates at pH 1.0, while the non-color hemiketal is present at pH 4.5 allowing the accurate and fast determination of total anthocyanins, still with the presence of polymeric pigments and other compounds (Wrolstad *et al.*, 2005). Briefly, 1 g of the plant material was extracted with the appropriate solvent and diluted with buffer solutions at pH 1.00 and pH 4.5. The difference in the absorbance at 510 and 700 nm at the different pH was used to calculate the monomeric anthocyanin pigment present in 1 g of the plant material using the following equation:

$$\text{Total anthocyanins (mg/L)} = A \times MW \times DF \text{ x}/\sum\text{x } 1 \quad (1)$$

where A is absorbance = $(A_{510\text{nm}} - A_{700\text{nm}})\text{pH}1.0 - (A_{510\text{nm}} - A_{700\text{nm}})\text{pH}4.5$; MW is the molecular weight; DF is the dilution factor; Σ is the molar extinction coefficient ($L \times mol^{-1} \times cm^{-1}$).

Chronic toxicity tests. Thirty five male Charles Foster rats weighing between 116–179 g were divided into seven groups of five animals each. The animals were housed in the *in vivo* testing facility of the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, throughout the period of the experiment. They were maintained in standard environmental conditions (temperature 27 ± 1.5 °C, humidity 73 ± 2.3%) and allowed free access to feed and water *ad libitum*.

To each group was administered 300 mg/kg or 2000 mg/kg body weight of either the water (A), 50% ethanol (AE) or ethanol (E) extract daily with the aid of an oral feeding tube. The seventh group was the control and was administered with 2 mL of water daily with the feeding tube. The animals were weighed every 2 weeks and the food intake was monitored for the first 45 days of the study.

The animals were observed for any form of morbidity and/or mortality during the 90 day period. Every 4 weeks, 1.2 mL blood samples were taken from each animal and subjected to haematological and biochemical tests.

Biochemical tests. Plasma was obtained by centrifuging heparinized blood at 4500 rpm for 10 min. The plasma obtained was analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, triglycerides, creatinine, and high and low density lipoprotein cholesterol and using a RA-50 standardized Clinical Chemistry System (RA232C, Serial No 30650, Bayer Diagnostics Mfg. Ltd, Swords, Co. Dublin, Ireland).

Histopathological evaluation. The animals were killed at the end of the experiments. Organs such as liver, heart, spleen, lung and kidneys of the animals were collected and weighed to determine relative organ weights. Then 10 μm tissue slides of the liver, kidney and spleen were prepared using a Kryostat (Leica CM 1900, V5.OD, Leica Microsystems Nussloch GmBH, Germany). The tissues obtained were stained using haematoxylin–eosin and viewed under a microscope (Leica DM LB2, Model 11888110, Leica Microsystems, Wetzair, GmBH, Germany) to evaluate histopathological changes.

Statistical analysis. One-way analysis of variance, ANOVA, was used to analyse the mean values obtained for the treatment and vehicle groups. Dunnett's post-hoc test was used to compare the treatment and vehicle groups and statistical significance was set at $p \leq 0.05$.

RESULTS

The extract yield was found to be 18.54% with ethanol (E), 15.18% with water (A) and 15.0% in 50% ethanol (AE). Ethanol was observed to extract the least amount of anthocyanin (as cyanidine-3-glucoside) of 1.23 mg/g of plant material, while 50% ethanol and water extracted higher anthocyanins at 3.83 mg/g and 3.22 mg/g, respectively.

Softening of faeces with a colour tending towards black was observed with animals on 2000 mg/kg by 24 h post administration of the first dose of extract. By the end of day 3, all the animals on the 2000 mg/kg dose had developed gray tails with severe diarrhoea. By day 8, all the animals in the 2000 mg/kg of the water and absolute alcohol extract were dead, while animals in the 2000 mg/kg dose of 50% ethanol extracts experienced consistent loss of weight until the last animal died on day 28.

There was an increase in food intake (g)/kg body weight of animal/day in the water and ethanol 300 mg/kg extracts, whereas in the 50% ethanol extract, there was a comparably lower intake of food (Fig. 1a). The food intake was significantly different from the control (A300 day 15 $p < 0.05$; day 45 $p < 0.001$; AE300 day 30 and day 45 – $p < 0.001$ and E300 day 45 $p < 0.001$).

The animals on the aqueous extract showed a slight drop in weight by day 7. The greatest weight gain was observed after day 15 in all the groups except the ethanol 300 mg/kg (in which there was no weight loss). For the 50% ethanol extract, there was a weight increase up to day 7, after which there was a drastic loss of weight. The ethanol extract group exhibited a weight gain although the percent weight gained decreased with time. In all the groups, death in the animals was preceded by a severe loss in weight, which was not accompanied with diarhoea, except in animals in the 2000 mg/kg group (Fig. 1b).

Animals administered the lower dose of 300 mg/kg of water or 50% ethanol extracts exhibited loss of fur the first 3 days which continued until day 15. Animals in these groups, however, only showed slight diarrhoea with softening of faeces. There was total mortality in animals on 50% ethanol extract by day 40, and 80% animals in the water extract group died by day 60. No mortality was observed in animals on the ethanol extract.

In general, there was drop in the erythrocyte count of animals. The increase from 300 mg/kg to 2000 mg/kg

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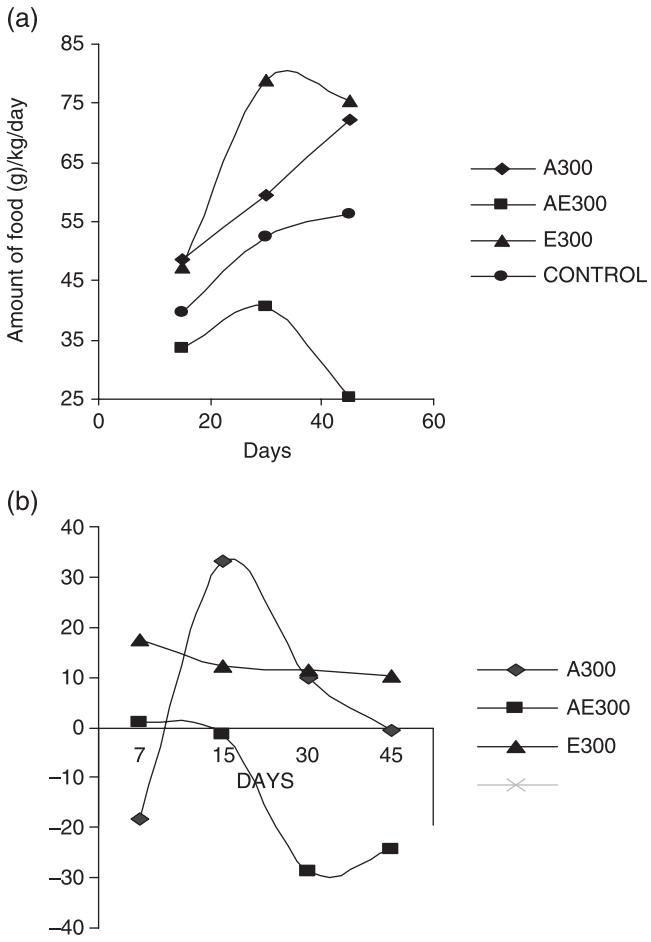

Figure 1. (a) Food intake per gram per day of animals on 300 mg and 2000 mg/kg body weight extracts; (b) Changes in weight gain of animals on 300 mg/kg body weight dose of aqueous (A), 50% ethanol (AE) and ethanol (E).

did not make any significant difference. A summary is shown in Table 1. There was no difference in the total leucocyte count (data not shown).

A summary of the biochemical parameters is given in Table 1. The activity of a liver function enzyme, aspartate aminotransferase, AST, was greatly enhanced by the administration of the aqueous and 50% ethanol extracts with a significant increase ($p < 0.05$) in activity with higher doses. The ethanol extract, however, had no significant effect on the activity of AST (see Table 1). After day 7, there was a drop in the AST level in most of the groups.

Alanine aminotransferase (ALT) was significantly affected by all the extracts at the different dose levels. The aqueous extract led to a significant increase ($p < 0.05$) in ALT levels. Administration of more doses led to a reduction in ALT levels. The effects observed in the higher dose were significant ($p < 0.01$). With the other extracts, the ALT level was significant regardless of the number of doses (Table 1). An increase in the dose of the 50% ethanol extract gave rise to significant changes in increase of the ALT levels, an effect not observed with the ethanol extract.

Creatinine levels were significantly affected by all the extracts, with a significant change with increase in dose of extracts for the water extracts ($p < 0.05$). There was however no significant difference between groups for the AE and E extracts ($p > 0.05$). In all the groups, an increase in the number of doses led to a reduction in serum creatinine levels.

Except for the AE 2000 mg/kg dose ($p < 0.01$), there was no significant change in the cholesterol and triglyceride levels. There was no difference in the lipid (high and low density).

There was no significant change in the sections of tissues of the heart and liver. In the control kidneys, however, the renal corpuscules were smaller and discrete while they were more widely spread in both the aqueous and ethanol groups. The relative organ weight is given in Table 2. There was a significant reduction in the weight of the spleen of the animals administered with ethanol and water extracts when compared with the control ($p < 0.01$). Other organs were of the same relative weight.

DISCUSSION AND CONCLUSION

This study shows that the continuous administration of high doses of the extracts of *Hibiscus sabdariffa* (10 000–66 000 fold of human consumption daily) for a prolonged period of time can lead to toxic reactions that may eventually lead to death. The increased level of liver function enzymes observed without changes in liver hepatocytes showed that the effects of the extracts may mimic chronic hepatitis without noticeable damage to hepatocytes. Also, the slight difference in these liver enzymes with an increase in the dose of extracts show that much damage may not be done with dose levels higher than 300 mg/kg in animal models. It is paradoxical that beverages made from this plant are folklorically used in liver disorders (Chen *et al.*, 2003) though at much lower doses. Previous studies have shown that the extracts of *Hibiscus sabdariffa* elevate liver function enzymes even after a few days of administration at doses of 150–180 mg which are lower than those used in this study (Akindahunsi and Olaleye, 2003; Ali *et al.*, 2005). It has been postulated that the higher the ratio of AST/ALT greater than 1 (Hawcroft, 1987), the higher are the chances of myocardial infarction. This ratio was not found to be significantly higher than the control in this study. Also, in the heart tissues microscopically, there was no difference between the control and treatment animals.

Prior studies showed the LD_{50} of the extract to be as high as 5000 mg/kg and some other reports have showed that dose levels as high as 4.6 g/kg can be administered in drinking water for as long as 12 weeks with no report of mortality (Orisakwe *et al.*, 2004), though with severe toxic effects on testes and sperm count in an animal model. A different experience was found in this study which may probably be due to two major factors, namely the method of extraction (solvents used, method of administration) and the different varieties of *Hibiscus sabdariffa* used in other studies. There have been reports showing different levels of anthocyanins in the different varieties of the plants in our laboratory (Pal, unpublished data). The maximum anthocyanin content was found in the hydroalcoholic extract followed by the aqueous and ethanol extracts. The lowering of triglycerides and the increase in RBC counts could be correlated with the content of anthocyanins in the sample.

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Table 1. Effect of the extracts of *Hibiscus sabdariffa* extracts on haematological and biochemical parameters

Parameter	Day	Treatment and dose administered			
		A300	AE300	E300	Control
RBC (10^6)	7	3.77 ± 0.65[b]	3.03 ± 0.07[b]	3.70 ± 0.36[b]	7.32 ± 1.553
	15	4.23 ± 0.29[b]	4.73 ± 0.45[a]	4.71 ± 0.58[a]	
	30	3.76 ± 0.11[b]	5.61 ± 0.44	3.57 ± 0.27[b]	
	45	5.08 ± 0.48	–	4.54 ± 0.28[a]	
	60	3.37	–	4.39 ± 0.54[a]	
	90	4.58	–	4.08 ± 0.68	
SGOT (AST) U/L	7	174.00 ± 6.57[a]	171.6 ± 13.96	144.6 ± 5.81	103.66 ± 2.75
	15	124 ± 12.96	100.8 ± 11.43	149.2 ± 10.78	
	30	156 ± 11.52	168.5 ± 38.74	136.2 ± 9.55	
	45	164 ± 3.65	–	154.4 ± 20.73	
	60	152	–	148.0 ± 10.33	
	90	148	–	143	
SGPT (ALT) U/L	7	70 ± 4.2[a]	72.4 ± 6.21[b]	80.0 ± 5.33[b]	38.55 ± 2.20
	15	62 ± 7.83	44.5 ± 5.38	72.0 ± 7.07[b]	
	30	57 ± 6.6	149 ± 40.9[b]	60.0 ± 13.03	
	45	54 ± 3.34		81.0 ± 7.0[b]	
	60	64		50.0 ± 3.46	
	90	48		71 ± 4.12[a]	
TRIG. mg/dL	7	58.68 ± 11.83	80.18 ± 6.22	62.00 ± 9.19	63.52 ± 6.32
	15	49.13 ± 11.73	51.1 ± 2.63	63.54 ± 5.58	
	30	49.83 ± 8.45	36.53 ± 5.53	61.26 ± 5.38	
	45	59.0 ± 20.35		50.38 ± 7.27	
	60	60		63.87 ± 5.65	
	90	66		56.37 ± 2.64	
CHOL mg/dL	7	97.02 ± 6.83	80.98 ± 4.19	82.48 ± 8.17	76.08 ± 8.45
	15	86.53 ± 7.13	107.12 ± 10.03	82.12 ± 9.69	
	30	67.43 ± 11.09	101.57 ± 18.95	80.00 ± 12.42	
	45	43.9 ± 6.53	–	49.78 ± 3.38	
	60	30.3	–	60.93 ± 7.92	
	90	43	–	56.1 ± 6.69	
H-DLC	7	59.52 ± 4.34	49.24 ± 6.57	61.52 ± 8.66	28.26 ± 3.77
	15	48.13 ± 12.91	52.68 ± 10.38	41.62 ± 4.80	
	30	34.68 ± 7.40	41.05 ± 11.25	40.44 ± 5.02	
	45	30.75 ± 3.04	–	47.63 ± 19.91	
	60	31.8	–	35.45 ± 5.16	
	90	32.5	–	37.22 ± 4.33	
CREAT mg/dL	7	2.59 ± 0.34[a]	2.98 ± 0.30[c]	3.05 ± 0.72[c]	0.84 ± 0.04
	15	2.97 ± 0.18[c]	2.47 ± 0.22[a]	3.29 ± 0.16[c]	
	30	3.97 ± 0.89[c]	2.61 ± 0.56[a]	3.69 ± 0.51[c]	
	45	1.13 ± 0.07		1.16 ± 0.04	
	60	1.2		1.10 ± 0.12	
	90	0.8		0.75 ± 0.03	
L-DLC mg/dL	7	25.80 ± 2.51	21.41 ± 8.57	22.19 ± 8.09	35.12 ± 1.63
	15	47.11 ± 10.69	41.75 ± 9.32	27.79 ± 13.01	
	30	22.72 ± 3.92	43.71 ± 8.07	32.84 ± 14.8	
	45				
	60				
	90				

A, aqueous extract; AE, 50% ethanol extract; E, ethanol extract.
[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.

Table 2. Relative organ weight after 90 day administration of extracts

Organ	Extract (percent relative organ weight)		
	Water	Ethanol	Control
Liver	3.93 ± 0.42	4.12 ± 0.09	4.08 ± 0.03
Spleen	0.35 ± 0.02[a]	0.48 ± 0.04[a]	0.68 ± 0.01
Heart	0.67 ± 0.23	0.44 ± 0.03	0.59 ± 0.01
Lungs	1.00 ± 0.37	0.78 ± 0.08	0.91 ± 0.06
Kidney	0.54 ± 0.05	0.46 ± 0.02	0.50 ± 0.01

[a] $p < 0.01$ when compared with control.

In this study, plasma creatinine was increased significantly by the administration of extracts in the first 30 days of the experiments with an increase in the number of doses, a factor which could be due to muscular dystrophy, or a loss of function of the kidneys. Also, a significant increased creatinine level in the serum beyond normal values has been associated with increased mortality (Gibson *et al.*, 2003). Ethanol extracts also caused a significant increase in the creatinine levels in this same group, though with no mortality, suggesting that the cause of death in the animals may not be due solely to the serum creatinine levels. An increase in serum creatinine

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was observed with the increasing dose level. This might have been caused by the drastic weight changes experienced by animals at the higher dose of 2000 mg/kg.

The total cholesterol and triglycerides of the treatment groups were not adversely affected except for animals in the AE 2000 mg/kg body weight. The HDL-C and the LDL-C values did not differ significantly with the control values. This confirmed studies carried out by Chen *et al.* (2003) that the water extracts of the plant reduces the level of cholesterol and triglycerides in animals fed with a high cholesterol diet. Though in this study, there was no reduction in the values at the early stage of the study, by the end of the 30 day of administration there was a significant reduction in the triglyceride level, while the cholesterol levels were always lower than the control values in most of the groups.

In conclusion, this study shows that very high doses of extracts of *Hibiscus sabdariffa* (over 10 000 human consumption) could be toxic to the hepatic system, and cause muscular dystrophy. Generally, the extracts obtained with three different solvents showed a different toxicity profile. The water extract, usually taken as a beverage, exhibited an ability to cause an increase in serum creatinine, while the extracts obtained with alcohol (absolute and 50%) had more damaging effects on the liver function enzymes in addition to an increase in plasma creatinine levels. The length of time of administration was not found to be particularly important since there was an obvious change in the biochemical parameters measured after 7 days of administration. Previous reports show that the extracts possess profound biological activities mainly antioxidant compared with standards but there is a need to investigate further the dose levels at which this activity is exhibited without toxic effects *in vivo*.

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