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Research Article

Aqueous Calyx Extract of *Hibiscus sabdariffa*: Impact on Growth, Gastrointestinal Morphometry, Liver and Clinical Chemistry of Suckling Rats

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Abstract

Background and Objective: The calyces of *Hibiscus sabdariffa* (HS) are consumed by all age groups in the tropics. The neonatal gastrointestinal tract (GIT) is susceptible to diet-induced structural and functional changes which may cause precocious maturation. The effects of an orally administered HS aqueous calyx extract on growth performance, gastrointestinal morphometry, liver lipid and glycogen content and clinical biochemistry were investigated. **Materials and Methods:** Forty-two, 4-day old Sprague Dawley rat pups from 4 dams were randomly assigned to groups and administered distilled water (10 mL kg^{-1}), a low dose (50 mg kg^{-1}) or a high dose (500 mg kg^{-1}) of HS via a stomach tube for 9 days. Intracardiac blood was collected and the viscera removed for gross morphometric measurements after euthanasia. Data was analysed using a one-way analysis of variance (ANOVA). **Results:** The low dose HS resulted in an increased tibial mass ($p<0.05$). The relative mass of the small intestine was significantly increased in the high dose (3.0±0.20% body mass) compared to the low dose group (2.7±0.22% body mass; $p<0.01$) and to the control group (2.7±0.27% body mass; $p<0.001$). The absolute and relative caecal masses of the pups on the high dose were heavier compared to those on the low dose ($p<0.05$). The HS aqueous calyx extracts did not alter activities of liver enzymes, hepatic glycogen and lipid content, nor change blood urea nitrogen and creatinine levels. **Conclusion:** *Hibiscus sabdariffa* (HS) intake by neonates may have trophic effects on the gastrointestinal tract without negatively affecting the health of the rat pups, the implications of which may result in increased food utilization efficiency later in life.

Key words: Gastrointestinal tract, *Hibiscus sabdariffa*, neonate, intestinal growth, tibial length

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hibiscus sabdariffa L., commonly called red sorrel, is a tropical wild plant belonging to the *Malvaceae* family[1,2]. It is thought to be native to Asia and/or Africa[1] but it is now cultivated in many tropical and subtropical regions of the world[3]. The fleshy flowers and calyces are processed into a refreshing beverage which is consumed hot or cold[4,5].

Previous phytochemical studies on HS have reported the presence of phenolics, organic acids, sterols, terpenoids, polysaccharides and minerals[6,7]. The phenolic content of the plant consists mainly of anthocyanins which have been shown to have antioxidant activity[6]. This antioxidant property is beneficial in providing protection from peroxidative damage in living systems[8].

In folk medicine, HS has been used as an antihypertensive, antiseptic, aphrodisiac, astringent, digestive aid and as a remedy for abscesses and heart ailments[9].

A lot of the folkloric claims about HS have been verified scientifically. For example, HS has been confirmed to have antihypertensive properties in animals[10,11] and in people[11-13]. It has been postulated that the antihypertensive action could be ascribed to a direct vaso-relaxant effect[14], sympathetic nervous system dependent mechanisms[15] and via inhibition of angiotensin converting enzyme by the anthocyanins in the plant's calyx extracts[16]. *Hibiscus sabdariffa* (HS) has also been shown to have hypocholesterolaemic[17-19], diuretic[20,21] antispasmodic[22] and anti-cancer activity[23,24]. Its extracts also have anti-pyretic[25], antimicrobial[26-28] and neuroprotective properties[29,30]. Previously showed that neonatal administration of HS aqueous calyx extracts to male and female rats protected them against fructose-induced hypertriglyceridaemia, increased liver lipid deposition (females) and hypercholesterolaemia (males)[31]. HS improved obesity associated nephropathy by improving glomerular filtration rate and lipid profile in an obese rat model[32].

The seeds of HS are traditionally used to induce lactation[33,34] and therefore, neonatal exposure can occur either directly when mothers give their babies HS to drink or indirectly via their breast milk. Like other nutraceuticals, the route of intake of HS is oral and therefore, it comes directly in contact with the gastrointestinal tract (GIT). The GIT serves as a connection between the diet and the metabolic processes that sustain life[35,36]. The structure of the neonatal GIT is susceptible to change in response to constituents of the diet[37,38]. Many peptides that regulate metabolism and digestion originate from the GIT[39-41]. Some plant extracts have been shown to cause precocious structural, maturational and functional changes in the GIT of especially neonates where permeability is quite high[42-45]. The suckling rat is thus an ideal model for neonatal studies on the morphological and functional effects of dietary modifications[44].

The objective of this study was to investigate the effects of an orally administered aqueous calyx extract of HS on the growth performance, GIT of suckling rats and biochemical markers of health.

MATERIALS AND METHODS

This study was undertaken from January-March, 2014 in compliance with the institutional guidelines for ethical research and animal welfare following approval of the protocol by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg (Certificate reference number: AESC/2013/46/05).

***Hibiscus sabdariffa* calyces: source, identification and extraction:** Dried calyces of HS were bought at the Central market in Sokoto, North Western Nigeria. They were identified by Mr. Halilu E. Mshelia of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto and a voucher specimen was deposited at the herbarium (PCG/UDUS/Malv/0001). The calyces were then transported to the University of the Witwatersrand, Johannesburg, Republic of South Africa, where the animal studies were carried out.

The dried calyces were ground to a fine powder using a blender (Waring®, Lasec, SA Company, USA). Two hundred and ten grams of the calyx powder were extracted in 1400 mL of distilled water at 95°C for 2 h[18]. The extracted solution was then filtered through Whatman No. 1 filter paper. The filtrate was concentrated using a rotor evaporator (Labocon (Pty) Ltd., Krugersdorp, Transvaal, South Africa). The extracts were then dried in an oven (Salvis®, Salvis Lab, Schweiz, Switzerland) at 40°C[43] and the residual powder extracts were collected and stored in dark, tightly sealed glass vials at 4°C for future use[18,46]. The yield was 22%.

Animals and experimental design: Four Sprague-Dawley rat dams that had given birth to a total of 42 (21 males and 21 females) suckling pups sourced from the Central Animal Services, University of the Witwatersrand were used in the study. Each dam and its litter were housed in Perspex cages lined with wood shavings. The dams were supplied with standard commercially sourced rat cubes (Epol, South Africa)

and tap water *ad libitum.* Ambient temperature was kept at 26±2°C and 12 h light cycles (lights on from 07h00-19h00) were followed.

The birth day of the pups was designated as day zero[44] and on day 4, the pups in each litter were randomly assigned to 3 treatment groups (split litter model). The rats in their respective treatment groups were distinctly identified using non-toxic markers on their tails. The 1st group which served as the control group (n = 14) received distilled water at 10 mL kg^{-1} body mass, the 2nd group (n = 14) received a low dose of HS extract (50 mg kg^{-1}) while the 3rd group (n = 14) received a high dose of the extracts (500 mg kg^{-1}). These doses are within the range used by other researchers without adverse effects on rats[33,47,48]. Based on the studies which have shown HS extracts to be of very low toxicity, a toxicity test was not done in this study. The treatments were administered intragastrically once daily in the mornings (08:00-09:00 h) using a 20G polyethylene tube mounted on a 1 mL syringe and passed orally[42-44] for 9 consecutive days till post-natal day 14. The treatment did not go beyond postnatal day 14 to prevent the compounding effects on the HS treatment that could result due to exploratory feeding of the pups after they open their eyes. The pups were weighed daily to adjust the amount given to maintain a fixed dose rate and also to monitor their growth performance

Terminal procedures: One day after the termination of the treatments, blood for glucose concentration determination was obtained from the pups via a pin prick to the tail before the pups were euthanased by intraperitoneal injection of sodium pentobarbitone (150 mg kg^{-1}, Euthapent, Kyron laboratories, South Africa). Blood was collected by cardiac puncture using 21G needles on 2 mL syringes into heparinised tubes. The blood samples were centrifuged at 4000 rpm at 4°C in a SorvallRT 6000B centrifuge (Du pont, USA) for 15 min and the plasma was collected and stored at -20°C until biochemical parameters were assayed.

The viscera were removed for gross morphometric measurements. The contents of the GIT were gently removed and the length and masses of the empty components were measured[42,43]. The other abdominal viscera were also weighed. The right hind limbs of the carcasses were carefully removed and the femur and tibia were cleaned of all flesh with a scalpel blade and pair of scissors. They were then dried in an oven at 50°C for 7 days until their dry mass was constant and then their lengths and weights were measured to determine linear growth and their mass, respectively.

The stored plasma samples were used to determine clinical biochemical surrogate markers of health using a calibrated colorimetric chemistry analyser (IDEXX Vet Test, Netherlands).

Hepatic storage of lipids was determined by solvent extraction as described by Bligh and Dyer[49] while hepatic glycogen stores were measured indirectly by acid hydrolysis to glucose as described by Passonneau and Lauderdale[50].

Statistical analysis: Data were analysed using Graph pad prism 5.0 (Graph pad, San Diego, CA) and expressed as mean±standard deviation. A one-way analysis of variance (ANOVA) was used to analyze the data between the groups, followed by a Bonferroni *post-hoc* test to compare the means. The significance level was set at $p \leq 0.05$.

All the animals remained healthy throughout the study and no incidental or iatrogenic morbidities or mortalities were recorded.

RESULTS

There was no significant difference among the treatment groups in both the induction and termination masses. Although all the pups increased in body mass significantly ($p<0.001$) from induction to termination (Fig. 1), there was no statistically significant difference in percentage body mass gain across the treatment groups (Fig. 2).

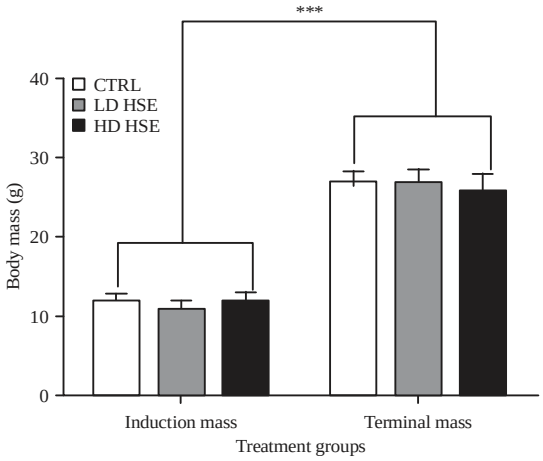

Fig. 1: Induction and terminal masses of rat pups in the different treatment groups

*** = p<0.001, Terminal vs Induction mass. HD HSE = High dose of *Hibiscus sabdariffa* extract (500 mg kg^{-1}), LD HSE = Low dose of *Hibiscus sabdariffa* extract (50 mg kg^{-1}), CTRL = Control. Data expressed as Mean±SD, n = 14 in each group

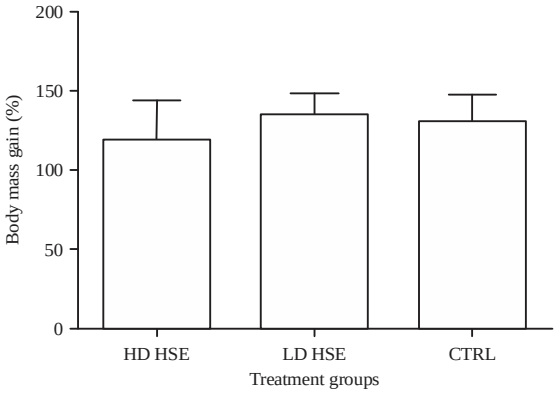

Fig. 2: Effect of HS on the body mass gain (%) of suckling rats after 9 days of treatment

HD HSE = High dose of *Hibiscus sabdariffa* extract, LD HSE = Low dose of *Hibiscus sabdariffa* extract, CTRL = Control. Data expressed as Mean±SD, n = 14 in each group

Table 1: Lengths, masses and density of tibiae and femora of suckling Sprague Dawley pups after 9 days of HS administration

Bones mass and length	CTRL	LD HSE	HD HSE
Tibia (mg)	32.0±3.50[a]	34.0±3.50[b]	30.0±4.60[a]
Tibia (mm)	16.0±0.95[a]	15.0±1.20[a]	15.0±1.40[a]
Tibia (mg mm)	2.0±0.15[a]	2.9±0.28[b]	2.0±0.23[a]
Femur (mg)	32.0±3.20[a]	32.0±3.40[a]	30.0±2.80[a]
Femur (mm)	12.0±0.72[a]	12.0±0.67[a]	12.0±0.42[a]
Femur (mg mm)	2.7±0.20[a]	2.7±0.22[a]	2.6±0.22[a]

[ab]with in rows, means with different superscripts are statistically different from each other at p<0.05. Pups in the LD HSE group had significantly heavier tibiae (p<0.05) compared to those of the other groups. Pups in the LD HSE had denser tibiae than pups in the HD HSE and CTRL groups. CTRL: Control (10 mL kg^{-1} distilled water), HD HSE: High dose of *Hibiscus sabdariffa* extract (500 mg kg^{-1}), LD HSE: Low dose of *Hibiscus sabdariffa* extract (50 mg kg^{-1}). Data expressed as Mean±SD, n = 14

Table 2: Effects of aqueous calyx extracts of HS on the absolute (g) and relative masses (% body mass) of the GIT and the liver and lengths (mm) of the small and large intestines of suckling rats

GIT, liver mass and length	HD HSE	LD HSE	CTRL
S.I.(g)	0.77±0.09	0.72±0.06	0.71±0.07
S.I. (% BM)	3.0±0.20[a]	2.7±0.20[b]	2.7±0.30[b]
S.I. (mm)	509±30.00	517±43.00	498±27.00
L.I. (g)	0.11±0.01	0.10±0.01	0.10±0.01
L.I. (% BM)	0.42±0.05	0.40±0.05	0.39±0.05
L.I. (mm)	70±5.30	72±4.70	71±4.80
Liver (g)	0.76±0.06	0.79±0.07	0.80±0.06
Liver (% BM)	3.0±0.14	2.9±0.17	3.0±0.17
Stomach (g)	0.17±0.02	0.16±0.02	0.17±0.02
Stomach (% BM)	0.66±0.07	0.59±0.07	0.63±0.08
Caecum (g)	0.062±0.02[ab]	0.049±0.01[c]	0.056±0.01[ac]
Caecum (% BM)	0.24±0.06[ab]	0.19±0.04[c]	0.21±0.05[ac]

Data in same row with different superscripts indicate significant differences at p<0.05. BM: Body mass, S.I: Small intestines, L.I: Large intestines, HD HSE: High dose of *Hibiscus sabdariffa* extract, LD HSE: Low dose of *Hibiscus sabdariffa* extract, CTRL: Control. Data expressed as Mean±SD, n = 14 in each group

Table 3: Effects of aqueous calyx extracts of HS on the hepatic storage of lipids and glycogen

Parameters	HD HSE	LD HSE	CTRL
Liver lipids (% liver mass)	8.8±4.8	8.1±3.9	8.4±4.8
[#]Liver glycogen (mmol L^{-1})	4.3±0.89	3.7±1.2	3.9±0.72

HD HSE: High dose of *Hibiscus sabdariffa* extract, LD HSE: Low dose of *Hibiscus sabdariffa* extract, CTRL: Control. [#]Glycogen is expressed as glucose equivalents in liver homogenate. Data expressed as Mean±SD; n = 14 in each group

Table 4: Effects of aqueous calyx extracts of HS on the clinical chemistry of suckling rats

Parameters	HD HSE	LD HSE	CTRL
Glucose (mmol L^{-1})	6.9±1.10	6.2±0.94	6.4±1.30
BUN (mmol L^{-1})	4.0±1.20	3.8±0.67	3.8±0.94
Creatinine (µmol L^{-1})	19.0±9.70	21.0±12.00	18.0±6.10
Phosphate (mmol L^{-1})	2.7±0.23	2.9±0.21	2.9±0.29
Calcium (mmol L^{-1})	2.5±0.24	2.3±0.36	2.4±0.53
Total protein (g L^{-1})	40.0±2.90	40.0±2.80	39.0±4.80
Albumin (g L^{-1})	16.0±5.60	15.0±5.90	16.0±4.70
Globulin (g L^{-1})	24.0±3.40	25.0±4.90	23.0±1.10
ALT (U L^{-1})	44.0±24.00	59.0±56.00	50.0±36.00
ALP (U L^{-1})	438.0±65.00	425.0±76	382.0±67
Total bilirubin (µmol L^{-1})	9.2±3.40	10.0±5.70	10.0±3.60
Cholesterol (mmol L^{-1})	4.4±0.30	4.4±0.28	4.1±0.32
Amylase (U L^{-1})	1347.0±173.00	1349.0±162.00	1272.0±254.00

BUN: Blood urea nitrogen, ALT: Alanine transaminase, ALP: Alkaline phosphatase HD HSE: High dose of *Hibiscus sabdariffa* extract, LD HSE: Low dose of *Hibiscus sabdariffa* extract, CTRL: Control. Data expressed as Mean±SD, n = 14 in each group

There was no significant difference in the masses of the long bones, however, the pups in the LD HSE had significantly heavier and denser tibiae (p<0.05) than pups in the control and the HD HSE groups (Table 1).

A significant increase was observed (p<0.001) in the masses of the small intestines relative to the body masses of suckling rats fed with a high dose extract of HS compared to the control group and also between the high dose and the low dose groups (p<0.01) as shown in Table 2.

There was no significant difference in percentage hepatic lipid and hepatic glycogen content after 9 days of treatment with HS (Table 3).

There was no significant difference in the clinical biochemistry surrogate markers of health measured between the different treatment groups (Table 4).

DISCUSSION

The aqueous calyx extracts of HS differentially affected the masses of the different segments of the intestines. There was an increased mass of the small intestines relative to the body mass in the HS groups when compared to the control group. This suggests that the HS extracts had a trophic effect on the small intestines of the rat pups. This is an important finding because to the knowledge, no studies that involve

direct administration of HS to neonatal age group have been done despite the obvious risk of potential exposure to this commonly used plant. Previous studies have shown HS aqueous[51] and methanolic[52] extracts to increase intestinal transit time and inhibit intestinal motility in adult rats. The flavonoids quercetin and eugenol were postulated to be responsible for these effects via modulation of Ca^{2+} channels[52]. The increase in the relative mass of the small intestines observed in the rat pups might be due to interference with digestion and absorption by HS extracts leading to accumulation of nutrients and subsequent stretching of the intestinal tissues[53]. Linderoth *et al.*[45] found that administration of red kidney bean (*Phaseolus vulgaris*) lectin caused an increase in small intestinal growth and number of crypt cells among other findings and concluded that it induced enhanced growth and precocious maturation of the GIT. Functional studies of the small intestines would have revealed whether HS extracts administered in the suckling period have any effect on the functional maturation of the small intestines.

In the present study, the absolute and relative caecal masses (relative to the body mass) of the high dose and low dose HS groups were also found to differ significantly from each other ($p<0.05$) but not from the control group. An increase in the caecal mass usually occurs following the introduction of a variety of substances into the GIT and is thought to result from the osmotic activity of substances not absorbed by the small intestines or an alteration of the GIT flora by dietary changes[44,54,55]. One would expect the difference to occur between the treatment groups and the control group. This difference though statistically significant, might therefore not be of any biological significance. Unfortunately, histology of the small intestines and caecum was not done and therefore the reported increase in their masses could not be attributed to either hyperplasia or hypertrophy. There was no significant difference observed in the biochemical surrogate markers of general health measured between the groups. Chronic administration of very high doses of HS has been associated with an elevation of the activity of some liver enzymes[9]. However, the administration of HS in the present study was for a comparatively short duration (9 days) and the doses used were also within the range used by other researchers without recording any adverse effects on liver or renal functions[33,47,48]. Plasma levels of alkaline phosphatase (ALP) were however elevated above the upper limit of 370 U L^{-1} for adult rats[56] in all the treatment groups. Plasma ALP activity is not specific for liver derangements because it arises from multiple sources including the bones, intestines, tumours and placenta[57]. Since the plasma activity of alanine transaminase and total bilirubin, total protein and albumin concentrations are essentially normal, the elevated ALP activity might be due to the osteoblastic and intestinal changes associated with growth[58].

Hibiscus sabdariffa extracts were shown to ameliorate acetaminophen-induced hepatic damage by increasing the level of glutathione and decreasing the level of lipid peroxidation[59] and protect the liver against CCL_4-induced liver fibrosis[60,61]. This hepato-protective activity of HS may explain why both the metabolic and synthetic functions of the liver were not affected.

There was no difference in the percentage body mass gain across the treatment groups ($p = 0.0747$). However, the HD HSE (500 mg kg^{-1}) had the least body mass gain of the three groups. HS has been reported to reduce bodyweight and reduce food intake[62]. The high dose HS may therefore have caused the seemingly reduced percentage weight gain in the HD HSE treatment group. The morphometric characteristics of the tibiae and femora are more reliable indices of growth because they are directly related to growth hormone and IGF-1 activity[63,64]. The lengths and density (as computed) of the tibiae of pups in the low dose HS group were significantly different from those of the control and high dose HS groups (Table 1). HS has about 12.63 mg/100 g calcium[1] which might contribute to the mineralization of the long bones. However, since the effect is only seen in the low dose HS and not the high dose HS group, there is need for further investigation of the mechanisms.

CONCLUSION

The findings from this study provide evidence that short term administration of aqueous calyx extract of HS might cause precocious maturation of the GIT without negatively affecting the general health of the neonatal rat.

SIGNIFICANCE STATEMENTS

This study discovers the possible trophic effects of HS on the neonatal gastrointestinal tract without negative consequences on the general health that can be beneficial for increased food utilization in later life. This study will help the researcher to know the consequences of early intake of HS drinks in neonates which researchers have not yet explored. Thus, a new theory on the safety or consumption of HS by the neonatal age group may be arrived at.

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