Food Research 8 (5) : 225 - 236 (October 2024)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

RESEARCH PAPER

Effects of the aqueous extract of *Hibiscus sabdariffa* on male fertility and reproductive performance in the rat model

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Article history:
Received: 3 July 2023
Received in revised form: 11 October 2023
Accepted: 28 August 2024
Available Online: 29 September 2024

Keywords:
Hibiscus sabdariffa,
Male fertility,
Reproductive performance,
Testosterone,
Sperm count

DOI:
https://doi.org/10.26656/fr.2017.8(5).211

Abstract

Hibiscus sabdariffa is a popular herb and has long been widely utilised for ethnomedicinal purposes. Despite its benefits, there is very scarce information available on the reproductive toxicity of this plant. The present study aimed to investigate the potential effects of the aqueous extract of *H. sabdariffa* (AEHS) on the male reproductive system of Sprague Dawley rats. A total of forty males were administered with AEHS at the different dosages of 250, 500, 1000 mg/kg/day or distilled water (control), by oral gavage daily throughout the 60-day treatment periods, which comprised three phases: pre-mating, mating, and post-mating. Results obtained demonstrated that the effects of AEHS on the reproductive system of male rats were slightly significant for certain doses. No mortality and any signs of physical and behavioural toxicity were observed. The mating performance was also not affected. Similarly, the mean body weight of rats was statistically not affected. However, the reproductive organ weights were found to be considerably different. Furthermore, AEHS increased the testosterone levels and sperm counts of the 250 and 1000 mg/kg dose groups, while the 500 mg/kg dose group showed considerably low levels for both parameters. The 500 mg/kg dose group was detected to exhibit inconsistent data for several parameters when compared to other groups, which might be caused by confounding factors instead of AEHS. Therefore, the current data suggest that AEHS should be consumed with caution particularly when the daily dose exceeds 250 mg/kg of body weight.

1. Introduction

Herbs have been extensively utilised by human beings as medications for a long time. The most popular reason for people to opt for natural sources as the cure for certain diseases is that they are believed to have little to no side effects (Zhang *et al.,* 2015). Most research in herbal medicines seeks to clarify their phytochemical frameworks (Tanaka and Kashiwada, 2021) and to identify the efficacy, mechanism of action, and toxicity characteristics (Chikezie and Ojiako, 2015). Due to proven herbal medicinal research over the years, paradigm changes in the 21st century toward therapeutic standardisation of herbal medicine have been started. Numerous *in vivo*, *in vitro*, and clinical investigations have verified and supported the efficacy and safety of herbal medicines (Ahmad Khan and Ahmad, 2019).

Hibiscus sabdariffa Linn, popularly known as roselle, is an annual herbaceous shrub belonging to the Malvaceae family, that originated from West Africa and was cultivated in Malaysia around the 1990s. Asam kumbang, asam susur and asam paya are among the vernacular names of roselle in Malaysia. It is an eco-friendly plant that can be grown within a short duration and can be found scattered around the tropical and subtropical climatic regions (Mohamad *et al.,* 2011). Currently, *H. sabdariffa* is extensively commercialised in the market as food-based products, beverages, sauces, wines, natural dyes, preserves and dietary supplements due to its promising therapeutic values (Wu *et al*., 2018; Salami and Afolayan, 2021). This plant is usually favoured for its calyx, flower, leaves, and seeds (Xie *et al.,* 2019) to be used as traditional medicines for treating a common cold, toothache, headache, indigestion, kidney and urine bladder stones and many more (Maganha *et al*., 2010). It contains abundant phytochemicals that are therapeutically beneficial to humans. These include unique constituents for instance, alkaloids, anthocyanins, carbohydrates, cardiac glycosides, flavonoids, gums, minerals, phenols, polyuronides, energy, carbohydrate,

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

protein, fat, reducing sugar, saponins, tannins, vitamins, essential and volatile oils (Islam, 2019; Salami and Afolayan, 2021). These contents have been described to possess pharmacological properties including antihypertensive (McKay *et al.,* 2010), antihyperlipidemic (Ali and El-Anany, 2017), cardioprotective (Lislivia Yiang *et al.,* 2017), antioxidants (Wu *et al.,* 2018), anticancer (Izquierdo-Vega *et al.,* 2020), anti-obesity, and hepatoprotective (Al -Snafi, 2018) effects. Moreover, other aspects of this plant that have been intensively researched are for antiviral, antidiabetic, cardiovascular, lipid profile, smooth muscle, wound healing, gastrointestinal, urinary, reproductive and central nervous systems (Singh *et al.,* 2017).

Despite the availability of extensive research on *H. sabdariffa*, studies on the possible toxicity of this plant on the male reproductive system are still scarce and remain inconclusive. Previous animal experiments on this plant generally reported beneficial pharmacological reactions, but the findings on the male reproductive system are variable. According to Ali *et al.* (2012), *H. sabdariffa* has been reported to exhibit an aphrodisiac and oestrogenic effects in male rats. In addition, this herb was found not to affect the male reproductive organs such as the testis, epididymis, ventral prostate, and seminal vesicle. In contrast, there is a study revealed that this herb was associated with short-term increases in circulating prolactin and testosterone, but lowered circulating FSH levels in male rabbits (Omotuyi *et al.,* 2010).

Due to the inconsistency of these previous findings, this present study was therefore carried out to investigate the potential effects of an aqueous extract of *H. sabdariffa* (AEHS), particularly from the calyces part on the male reproductive system of Sprague Dawley rats. This current study was conducted by adapting the Organization for Economic Cooperation and Development (OECD) test guidelines No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (OECD, 2015). Scientific information on the potential toxicity of this plant is crucial for safeguarding human exposure, enhancing product reliability and regaining trust among consumers. The calyces of *H. sabdariffa* are the most commonly consumed part because they contain high amounts of phenolic compounds such as anthocyanins, mainly delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside (Hassan *et al.,* 2015). The aqueous extract and oral route of administration were chosen for this study to reflect the traditional usage of this extract commonly utilised by the local populace.

2. Materials and methods

2.1 Plant materials

The calyces of *H. sabdariffa* were supplied by HERBagus Sdn. Bhd, Pulau Pinang as our research collaborator. The sample was then authenticated by a botanist from the Forest Research Institute Malaysia (FRIM). A voucher specimen No. PID 050515-05 was deposited at the Herbal Medicine Research Centre (HMRC), Institute for Medical Research, Malaysia.

2.2 Plant extraction

The AEHS was prepared using a sonication method in the HMRC, Institute for Medical Research, Kuala Lumpur, Malaysia. Fresh *H. sabdariffa* calyces (1 kg) with seeds removed (Figure 1 (A-B)) were washed with water and air dried. The dried samples were ground to a fine powder using a universal cutting mill. The extraction procedure was divided into three cycles to achieve the best and highest yield. In a sonicator, each cycle was run for 30 mins at 25°C. The *H. sabdariffa* powder was mixed with distilled water and subsequently heated until it reached 80°C because hot water appeared to be an effective solvent capable of producing over 78% yield. In the three extraction cycles, the ratios of *H. sabdariffa* powder to distilled water volume were 1:10, 1:7, and 1:4 respectively. The extraction was filtered using a tea filter sock after each cycle, and the total filtrates from all three cycles were frozen overnight at -20°C in the freezer. The frozen filtrate was then freeze-dried for two days. Finally, the yield was collected and stored in a bottle at 4°C until required. The authenticity of AEHS was confirmed by the presence of two essential marker compounds; delphinidn-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside which are anthocyanin derivatives by HPLC profiling (Maizatul Hashima *et al.,* 2018).

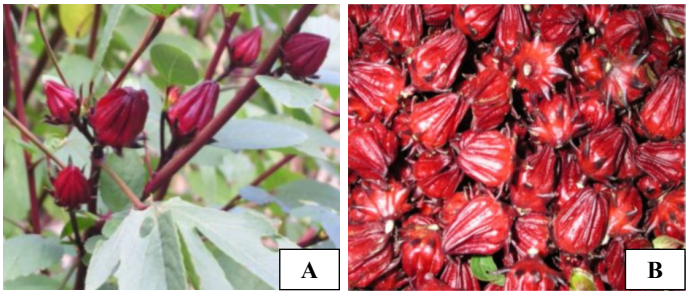

Figure 1. (A) The flowering stage of *H. sabdariffa,* (B) Fresh-ly harvested of *H. sabdariffa* calyces (Pegu *et al.,* 2021).

2.3 Dosage calculation and preparation

The selection of doses for this study was based on the pharmacological dose (300 mg/kg) of AEHS reported in previous investigations on rat models conducted by the same research team (Maizatul Hashima *et al.,* 2018; Nursyuhana *et al*., 2018). This was set at three different

https://doi.org/10.26656/fr.2017.8(5).211

dosages by manipulating the pharmacological dose to discover the optimal dose range involving a low, medium and high dose for *in vivo* experiment. In addition, the OECD guideline No. 422 (OECD, 2015) also recommends that the doses of the test substance for toxicity study should be at least 3 different dose levels. Thus, the highest concentration, 1000 mg/kg AEHS was chosen as a maximum limit dose for oral consumption in accordance with the OECD guidelines. The medium and low doses were selected at 500 and 250 mg/kg respectively, while distilled water was used as vehicle control.

Prior to oral dosing, the AEHS powder was weighed and reconstituted with distilled water to produce the three aforementioned doses and kept in labelled aliquots. The prepared doses were stored in the refrigerator at -20°C until use.

2.4 Animal husbandry and maintenance

The approval for this study was obtained from the USM Institutional Animal Care and Use Committee (IACUC) with the reference number: USM/Animal Ethics Approval/2020/(124)(1083). Forty male Sprague Dawley (SD) rats at the age of 2-3 months old, weighing between 230-290 g were procured. The selection of this range of age and weight was due to the suitable physiological and reproductive characteristics of fertile, young adults of this rat strain, as they reach young adulthood at approximately six weeks of age. Our rats of 2-3 months old are equivalent to 18.18-27.27 human years, based on the puberty formula of one human year equals 3.3 rat days (Sengupta, 2013). This age range could result in the most favourable reproductive outcomes across three major experimental stages of premating, mating and post-mating periods in this study. As compared to humans, rats have accelerated sexual maturity and adulthood, with the different phases of a rat's life providing an accurate correlation with human age (Siti Nurfarhana *et al*., 2022).

A substantial number of adult female SD rats were also used in this study for mating purposes. The selection of the SD rat was in accordance with the recommendations outlined in the OECD guidelines No. 422 (OECD, 2015), which advised utilising the same animal species employed in earlier investigations (Prommetta *et al.,* 2006; Sireeratawong *et al.,* 2013) that used the same test substance. The use of SD rats has long been approved for reproductive and developmental toxicity testing by international regulatory organisations. This sample size was also selected based on the same OECD guidelines which stated that the least number of animals required should be 10/sex in each group (OECD, 2015).

All animals were acquired from the Animal Research and Service Center (ARASC), Universiti Sains Malaysia Health Campus, Kelantan, Malaysia. They were kept individually in polypropylene cages at 23±1°C with 12 hrs of light and dark rotation in the controlled animal room. Standard rat pellets and tap water *ad libitum* were supplied for the animals. The chip-wood bedding was changed regularly to ensure proper hygiene and prevent the accumulation of ammonia and carbon dioxide which are detrimental to the health of the rats. The animals were permitted to be acclimatised for 7 days before the commencement of the study.

2.5 Experimental protocol and dose selection

The study was designed by adapting the OECD guidelines No. 422 (OECD, 2015). Forty SD rats (n = 10) were assigned to each dose level group; distilled water - control, AEHS 250, 500 and 1000 mg/kg body weight. The treatment volume of 0.7 mL each was delivered daily by gavaging during three main stages of the experiment which covered pre-mating, mating and post-mating periods. Throughout the study period, all animals were routinely monitored for changes in general physical health, behaviour, body weight, food consumption, mortality, morbidity, moribundity, or any signs of toxicity or abnormal occurrences were documented. Clinical signs and behavioural changes as well as body weight were recorded daily while food consumption was monitored weekly.

Following a 7-day acclimatisation period, the male rats were treated with AEHS for a 14-day pre-mating period (first stage). This stage was designed to observe the early impacts of AEHS dosing on the general physical health and behavioural signs of rats before mating. The AEHS administration was continued to the second stage, the mating period for a maximum duration of 14 days. In this stage, the treated male was placed together with an untreated, proestrous-stage female rat in a ratio of 1:1 to allow mating. The female's vaginal plug was examined for the presence of sperm the following morning. A sperm-positive vaginal smear indicated that mating had taken place and was designated as day zero (D0) of pregnancy. Apart from the mating process itself, mating performance was also evaluated based on these parameters: mating, libido and fertility indices. Following successful mating, both male and female animals were separated and returned to their original cages. The treatment for the males was continued to the subsequent stage (post-mating) for another 45 days. Generally, the overall treatment period of the males was at least 60 days depending on the length of time required for successful mating. The longer the days for successful mating, the longer the overall treatment period.

https://doi.org/10.26656/fr.2017.8(5).211

2.6 Animal dissection, blood withdrawal and macroscopical organ examination

The male rats that had completed the whole treatment period were ready to be sacrificed. All animals were fasted overnight starting in the evening of the last dosing. At the end of the experimental (D46 of post-mating) period, all animals were anaesthetised with 60 mg/kg sodium pentobarbital (Maizatul Hashima *et al.,* 2018) intraperitoneally. As soon as the rat achieved a state of anaesthesia, approximately 3-5 mL blood sample was collected via cardiac puncture. The heart of the rat was pushed slightly to the left to move it away from the sternum and the heartbeat was detected with the index finger. The syringe was placed at a 90-degree angle from the heart and the blood was slowly aspirated. It was stored in a 3 mL gel and clot activator blood tube for hormonal analysis.

Soon after the blood withdrawal, the animals were immediately laparotomised to allow macroscopical examination of the visceral organs i.e. reproductive organs, liver, kidneys, adrenal glands, spleen, stomach, intestine, heart, and lungs. Only the male reproductive organs such as the testes, epididymis, prostate gland and seminal vesicle were thoroughly inspected and resected from the body. The collected organs were cleaned by trimming the fat and adhering tissues, then placed in the correct right and left position in a petri dish. Each organ was finally weighed as soon as possible to avoid dehydration.

2.7 Sperm count analysis

Once the weighing process of the reproductive organs was completed, the cauda epididymis was isolated and moistened with a few drops of normal saline in a petri dish. The cauda epididymis was minced with sharp scissors in 2 mL of normal saline. The resultant mixture was filtered through 80 µm nylon mesh. Two drops of eosin Y were added to infiltrate the mixture and allowed to remain at room temperature for 15 minutes. The aliquot of the stained epididymal suspension was diluted 20 times with normal saline. The suspension was pipetted onto a Neubauer's chamber and viewed under a light microscope. The total sperm count was calculated in 8 squares of 0.1 cm^2. The calculation using the following formula was expressed as million/mL after correction for dilution (Hilmi *et al.,* 2015).

$$Sperm\ count\ (x) = x \times correction\ factor\ 5 \times 10^4$$

2.8 Reproductive hormone analysis

Animal blood samples obtained during laparotomy were subjected to the analysis of male reproductive hormonal levels. The blood was immediately despatched to the Gribbles Pathology Malaysia Sdn Bhd, Kota Bharu, Kelantan for analysis to ensure that it did not undergo haemolysis. The hormones tested were testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) because these are the ordinary and essential hormones responsible for optimal testicular performance and fertility in males (Zati Bayani *et al.,* 2022).

2.9 Outcome measures checklist

A considerable number of parameters were recorded throughout this study based on adaptation from the OECD test guidelines No. 422 (OECD, 2015). The outcomes checklist is outlined as follows:

i) Survivability

ii) Morbidity

iii) Moribundity

iv) Signs of toxicity

v) Behavioural changes

vi) Body weight

vii) Food consumption

viii) Days of successful mating

ix) Mating index = (No. of males mating / No. of males cohabited) x 100

x) Libido index = (No. of sperm-positive males / No. of males paired) × 100

xi) Fertility index = (No. of females pregnant /No. of sperm-positive females) × 100

xii) Absolute reproductive organ weight

xiii) Relative reproductive organ weight = (Organ weight/body weight) × 100

xiv) Sperm count

xv) Hormonal levels (FSH, LH, Testosterone)

2.10 Statistical analysis

All numerical data obtained in this study were analysed using GraphPad Prism Version 9 (GraphPad Software Inc La Jolla, CA, USA). All parameters were initially confirmed for normality using the Normality test as well as Levene's test to check for homogeneity of variance.

The normally distributed data were analysed using parametric tests; One-way ANOVA followed by Scheffe test when differences were found. Repeated observation data i.e. body weight and food consumption were tested using repeated measures ANOVA. In contrast, nonparametric tests; Kruskal Wallis followed by Mann-Whitney U test were used for skewed distribution as well as non-homogeneous data.

The parametric data were expressed as an arithmetic

https://doi.org/10.26656/fr.2017.8(5).211

mean ± standard error of the mean (SEM) while the non-parametric data were expressed as median (interquartile range) (IQR). The 0.05 level of probability was used as the criterion for significance and represented as different superscript letters.

3. Results

3.1 General health and behaviour

There were no treatment-related morbidity and mortality associated with the adverse effects in rats at any dose level tested throughout the administration of AEHS for approximately 60 days (Table 1). Furthermore, none of the animals exhibited abnormalities with regard to the skin, fur, eye colour, nasal discharge and faeces, locomotor progression and respiration. Their behaviour was also typical and comparable to those of normal control animals.

3.2 Mating performance

Table 1 summarises the data on the mating performance of the male rats throughout the study. Days of successful mating for male rats exhibited no significant differences among all experimental groups. Likewise, the parameters of mating and libido were comparable for all groups with 100% indices. Meanwhile, the fertility index showed a slightly lower value for the 1000 mg/kg treatment group (90%) compared to other groups which achieved 100% but no significant differences were noted.

3.3 Food consumption and body weight changes

Food consumption and body weight gain were routinely monitored in the test animals. The healthy patterns of food intake with the normal increment of body weight in all groups of animals were seen throughout the study. Statistically, no changes were noted in the food consumption trend among all experimental groups (Table 1). Corresponding to this, the increment of mean body weights in each group over time was also statistically not affected (Figure 2).

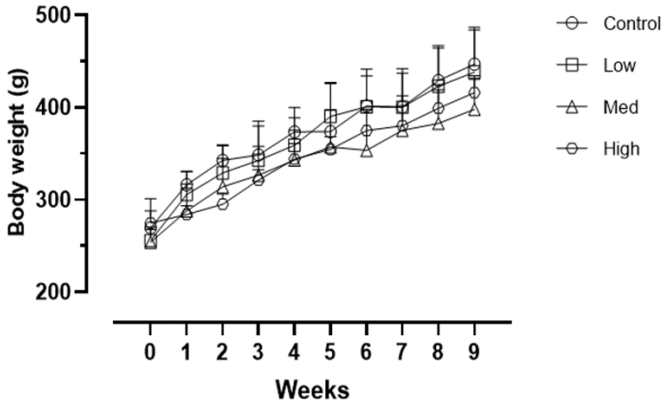

Figure 2. Body weight of male rats from weeks 1 to 9 of the experimental period. The mean body weight of all experimental groups increased with time during the experimental period. No significant differences (P>0.05, two-way ANOVA) were observed in this parameter for all experimental animals.

3.4 Gross examination of visceral organs

Overall, there were no abnormalities observed in the gross morphology of the male reproductive organs. There were no noticeable alterations on the organs examined macroscopically such as the seminal vesicles, prostate gland, epididymis, and testes between the AEHS -treated and the control animals.

3.5 Reproductive organ weights

Table 2 shows the effects of AEHS on the weights of male reproductive organs. For absolute testis weight, the medium-dose (AEHS 500 mg/kg) group was found to be significantly different (P<0.05) when compared to other groups including the control. As for relative testis weight (organ weight to body weight ratios), only the medium-

Table 1. Effects of AEHS treatment on survivability and reproductive performance in male rats.

Parameters	AEHS				*P*-value
	Control (n = 10)	250 mg/kg (n = 10)	500 mg/kg (n = 10)	1000 mg/kg (n = 10)	
Survivability (%) [a]	100±0	100±0	100±0	100±0	*P*>0.05
Morbidity (%) [a]	0	0	0	0	*P*>0.05
Final week food intake (g) [a]	28.93±1.38	29.22±1.41	28.79±1.36	29.23±1.33	*P*>0.05
Days of successful mating [b]	3.1 (4.5)	1.5 (0.5)	2.7 (3.25)	3.2 (3.75)	*P*>0.05
Mating index (%) [a]	100±0	100±0	100±0	100±0	*P*>0.05
Libido index (%) [a]	100±0	100±0	100±0	100±0	*P*>0.05
Fertility index (%) [a]	100±0	100±0	100±0	90±31.62	*P*>0.05

[a] Data were analysed by One-way ANOVA test. Values are presented as mean ± SEM.
[b] Analysed by Kruskal wall test. Values are presented as median (interquartile range, IQR).
Mating index = (No. of males mating / No. of males cohabited) × 100
Libido index = (No. of sperm positive animals/ No. of animals paired) × 100
Fertility index = (No. of females pregnant / No. of sperm positive females) × 100

https://doi.org/10.26656/fr.2017.8(5).211

Table 2. Effects of AEHS on absolute and relative weights of the reproductive organs as well as sperm count in male SD rats.

Parameters	AEHS				*P*-value
	Control (n = 10)	250 mg/kg (n = 10)	500 mg/kg (n = 10)	1000 mg/kg (n = 10)	
Testis					
Absolute (g)	1.636±0.062	1.677±0.092	1.075±0.203[abd]	1.755±0.122	*P*<0.05
Relative (%)	0.378±0.013	0.381±0.014	0.272±0.051[d]	0.432±0.030	*P*<0.05
Prostate					
Absolute (g)	0.783±0.032	0.543±0.078[a]	0.478±0.028[a]	0.639±0.034	*P*<0.05
Relative (%)	0.176±0.007	0.125±0.018[a]	0.121±0.007[a]	0.157±0.008	*P*<0.05
Epididymis					
Absolute (g)	0.646±0.023	0.797±0.027[a]	0.717±0.026	0.734±0.034	*P*<0.05
Relative (%)	0.145±0.005	0.185±0.011[a]	0.182±0.010[a]	0.181±0.008[a]	*P*<0.05
Seminal vesicle					
Absolute (g)	1.005±0.058	0.738±0.054[a]	0.697±0.044[a]	0.719±0.062[a]	*P*< 0.05
Relative (%)	0.226±0.013	0.171±0.015	0.179±0.016	0.177±0.016	*P*> 0.05
Sperm count (10^6)	7.66±0.61	9.71±0.83	4.57±0.51[abd]	8.20±0.66	*P*< 0.05

All data were analysed by One-way ANOVA test. Values are presented as mean ± SEM.
n = number of male rats, *P*< 0.05 significant difference.
Relative weight (%) = organ weight related to body weight.
[a] Significant difference in comparison with the control group. [b] Significant difference in comparison with the low (250 mg/kg) dose group. [c] Significant difference in comparison with the medium (500mg/kg) dose group. [d] Significant difference in comparison with the high (1000 mg/kg) dose group.

dose group (AEHS 500 mg/kg) differed significantly when compared to those of the high-dose (AEHS 1000 mg/kg) group.

The absolute and relative weights of the prostate glands in the control group exhibited a significantly higher value (*P*<0.05) when compared to the low- and medium-dose treatment groups. While for epididymis, absolute weight was significantly higher (*P*<0.05) for the low-dose treatment group as compared to the control animals. Meanwhile, for relative epididymis weight, all treatment groups showed significantly higher values when compared to the control group. For absolute seminal vesicles, there was a significant difference (*P*<0.05) between the control and all treatment groups. As for relative seminal vesicle weight, no significant difference was observed among all treatment groups.

3.6 Sperm count

The results of sperm count are summarised in Table 2. It was shown that daily oral administration of AEHS to male rats substantially increased the epididymal sperm count in male rats of low-dose (250 mg/kg) and high-dose (1000 mg/kg) groups, as compared to the control animals. In contrast, the medium-dose (500 mg/kg) group exhibited the significantly lowest sperm count when compared to other treatment groups.

3.7 Reproductive hormonal levels

There were no significant differences in the FSH and LH levels among all treatment groups. In contrast for testosterone level, the low (250 mg/kg) dose group displayed a significantly higher value when compared to the control and medium-dose (500 mg/kg) groups (*P*<0.05). Despite the medium-dose group having a slightly higher reading than the control group, it was not

Table 3. Effects of AEHS treatment on sex hormonal levels in male rats.

Parameters	AEHS				*P*-value
	Control (n = 10)	250 mg/kg (n = 10)	500 mg/kg (n = 10)	1000 mg/kg (n = 10)	
FSH (IU/L)	0.3±0	0.3±0	0.3±0	0.3±0	*P*>0.05
LH (IU/L)	0.3±0	0.3±0	0.3±0	0.3±0	*P*>0.05
Testosterone (ng/mL)	1.61±0.28	5.47±1.18[ac]	1.71±0.35	3.55±0.63	*P*<0.05

All data were analysed by One-way ANOVA test. Values are presented as mean ± SEM.
[a]Significant difference in comparison with the control group. [c] Significant difference in comparison with the medium (500 mg/kg) dose group.

https://doi.org/10.26656/fr.2017.8(5).211

statistically significant. Table 3 summarises all three (FSH, LH, and testosterone) levels across all experimental groups, indicating the reproductive hormonal fluctuations in male rats following oral administration of AESH.

4. Discussion

The popularity of *H. sabdariffa* in tropical regions has enticed scientific researchers to embark on various studies due to its diverse unique constituents with great medicinal potential. It has been shown to be generally safe for short and long-term use. To date, the *in vivo* general oral toxicity evaluations of *H. sabdariffa* have covered acute (Onyenekwe *et al.,* 1999), subacute (Prommetta *et al.,* 2006), subchronic (Mahfudh and Ikarini, 2018), and chronic (Sireeratawong *et al.,* 2013) exposures with no substantial negative impacts on crucial outcomes. Despite the availability of general toxicity studies, it is still inadequate to convince that *H. sabdariffa* is safe to be consumed as a complementary herbal medicine since the effects on male reproductive toxicity are still scarce with conflicting findings. It is therefore, the present study was conducted to determine the potential short-term toxicological effects of AEHS, particularly on the male reproductive system of the rat model, in order to justify its safety profiles for ultimate human use.

The current study utilised an aqueous extract of *H. sabdariffa* calyces because this type of extraction is safe, can extract polar compounds and mimic human practices in the traditional usage of this plant. Calyces are the most exploited part of the roselle and are obtained by removing the petals of its flower to produce various food products.

Administration of AEHS for approximately 60 days produced no treatment-related mortality, morbidity or aberrant physical characteristics associated with the adverse effect in rats at any dose level tested. Nevertheless, at certain points throughout the study period, there were slight fluctuations in body weight particularly those of the high-dose (1000 mg/kg) and medium-dose (500 mg/kg) groups. This was however, not statistically significant and could be due to the slight unpalatability of the highly concentrated solution of AEHS during the force oral feeding. Noviatri *et al.* (2020) disclosed that the average value of sour and taste of roselle increased as the concentration increased. Otherwise, the average value of its sweetness decreased as concentration increased following the hedonic test. Overall, the body weight progressions of AEHS-treated and the control animals were still within the normal range of Sprague Dawley rats of a similar age. In general, these findings indicate that AEHS did not adversely alter the parameter of body weight in male rats. It is well established that body weight changes have frequently been used to indicate the adverse effects of drug and chemical exposure (Talbot *et al.,* 2020).

One approach to examining the reproductive performance of male rats was to observe the days required for successful mating. This allowed us to make a general assumption on the effect of AESH on the mating capability of the rat. The lower the number of days required for successful mating, the better the result of a mating performance. In this study, there was no significant difference in the days taken for mating among all experimental groups, indicating that AEHS did not cause any alteration to the sexual motivation of the male rats. A mating index is a proportion of the number of males successfully mating out of all males cohabited, and expressed in percentage (Liu *et al.,* 2018). This study provides evidence that AEHS did not affect the mating index across every group. Every male who cohabited was successfully mated with a female. Apart from this, the libido index is used to observe the number of females with sperm positive out of the total number of females mated (Ochei *et al.,* 2017).

Similar to the mating index, the result confirmed that AEHS did not cause any significant sexual modification in the libido index of the male rats. A further parameter that we observed in this experiment was the fertility index. The fertility index is used to assess the effectiveness of a male's sperm to induce pregnancy (Abbas *et al.,* 2018). This parameter was observed by counting the pregnant females from those showing sperm -positive vaginal smears. The findings turned out that only the high-dose (1000 mg/kg) group animals showed 90%, a slightly decreased fertility index when compared to other groups that achieved 100%. However, this was not statistically significant since only one male out of 10 rats per group failed to impregnate a female rat. Reduced reproductive performance and fertility in males are caused by numerous factors such as the underdevelopment of reproductive organs, a decrease in the number of normal sperm, inhibited sperm motility, aberrant sperm motion and a decrease in acrosome response (Gao *et al.,* 2020). None of these characteristics were observed on the particular male rat as he looked healthy and active physically. Our findings are in agreement with previous studies by Ali *et al.* (2012) and Sireeratawong *et al.* (2013) reporting that *H. sabdariffa* calyces extract did not exert any significant detrimental effects on the male reproductive system at the highest dose of 200 mg/kg/day even used for a chronic period. In addition, an old subacute toxicity study by Prommetta *et al.* (2006) also suggests that the aqueous extract of this

https://doi.org/10.26656/fr.2017.8(5).211

plant at doses ranging between 200-1000 mg/kg/day demonstrated no adverse effects on the contents and activities of hepatic cytochrome P-450. The extract also did not show harmful effects on several important organs/systems such as the liver, kidney, blood system, electrolytes as well as lipid and carbohydrate metabolism. On the basis of the available evidence and the lack of statistical significance, we therefore deduce that AEHS had no substantial negative influence on the fertility index of male rats.

Organ weight changes are established as one of the most sensitive markers of a toxic test substance (Nirogi *et al.,* 2014). When analysing data on organ weight from animal experiments, it is standard practice to examine both actual organ weight (referred to as absolute organ weight) and organ weight represented as a percentage of body weight (referred to as relative organ weight) (Nirogi *et al.,* 2014). The relative organ weight is a trusted variable because it eliminates the confounding variations owing to body mass (Lazic *et al.,* 2020). Nevertheless, both absolute and relative organ weights are critical parameters because organ weight loss is possible and is not always associated with a reduction in body weight (Nirogi *et al.,* 2014). In this study, there were significant random variations in the absolute and relative weights for male reproductive organs such as the testes, prostate gland, epididymis, and seminal vesicles. There were no discernible trends in these organ weights making it difficult to determine their exact causes. However, these findings may suggest that AEHS affects male reproductive organs, which contradicts the available previous evidence (Ali *et al.,* 2012; Sireeratawong *et al.,* 2013).

For the testis, the high-dose group (1000 mg/kg) displayed the significantly highest values of both absolute and relative weights as compared to the lowest values by the medium-dose (500 mg/kg) group. However, the lowest testis weight in the medium-dose group appears to have randomly occurred, did not demonstrate a dose-dependent trend and therefore, could not be explained in detail which requires further investigation. At least, the effect seen on the medium-dose (500 mg/kg) group might be correlated with their triple effects of decreased testosterone level, sperm content and body weight at the same time. Arruda *et al.,* (2016) revealed that maternal exposure to *H. sabdariffa* at doses of 250 and 500 mg/kg can adversely influence the male reproductive system in rats by lowering the sperm count, but they did not observe for testis weight. Typically, a low sperm count could indirectly reduce the testis weight. In toxicology, the common attributable factors for reduced testis weight are low testosterone level, suppression of spermatogenesis and degeneration of testicular tissue (Kumar and Nagar, 2014). Our current study lacks histopathological information on the male reproductive organs, necessitating additional investigation to confirm the effects of AEHS on testicular weight.

Furthermore, the prostate glands of the low (250 mg/kg) and medium (500 mg/kg) dose groups also exhibited significant reductions in both absolute and relative weights when compared to the control animals. Previous studies had reported that the quantification of prostate gland weight is less useful because of smaller size and technical difficulties of consistent dissection (poor or unclear delineation), non-therapy-related physiological variables, low weight association with histopathological results, or unspecific weight loss due to reduced body weight (Michael *et al.,* 2007; Wolfsegger *et al.,* 2009). Meanwhile, the relative epididymis weight increased significantly across all treatment groups when compared to the control group. This finding may be due to an increase in testosterone levels. An impact caused by the increment in testosterone level should have resulted in an increase in the weight of the accessory organs. As a consequence, it is possible to correlate that the increased weight of the testis and epididymis was due to a combination of high testosterone levels and a high number of sperm stored in these organs (Morakinyo *et al.,* 2008). However, this fact does not apply to the medium-dose group since testosterone level and sperm count were both low but still showed an increase in the epididymis weight. This might be caused by an alteration in structure or content in the corpus or caput epididymis which possesses some protein synthesis properties (Marchiani *et al.,* 2017). Finally, only the absolute weight of the seminal vesicle showed a significant reduction for all treatment groups as compared to the control animals. Nevertheless, the relative weight of this organ showed no significant difference across all groups. This implies that the difference in the seminal vesicle progresses in the same way as body weight. It is well-established that some organ weights increase in accordance with body weight (Nirogi *et al.,* 2014).

Besides organ weights, the altered endocrine function of the reproductive hormone is one of the toxicological endpoints in the male reproductive toxicity study (Arzuaga *et al.,* 2019). Testosterone, a primary male sex hormone produced by the testicles, has several functions in the body. This includes regulating libido, bone density, muscle mass and strength, fat distribution and sperm production in males (Goymann and Wingfield, 2014). Reduced testosterone levels may be associated with reduced fertility. Data obtained from this study revealed that serum testosterone levels following daily administration of AEHS were elevated in all

https://doi.org/10.26656/fr.2017.8(5).211

treatment groups when compared to the control group. The low-dose group (250 mg/kg) demonstrated the significantly highest serum testosterone level when compared to the control and medium-dose group (500 mg/kg). The fluctuation trend remarked in this parameter is almost similar to that of testis weights. However, the FSH and LH levels of the male rats were low and minimally detected. The findings on the testosterone levels are in line with those of Omotuyi *et al.* (2010) in their earlier study. They suggested that the increase in the circulating testosterone level in males could be caused by the direct effect of anthocyanin from *H. sabdariffa* calyces extract on testicular steroidogenesis and feedback inhibition on FSH synthesis in the pituitary. They described that anthocyanin can be absorbed in the gastrointestinal tract as anthocyanidins, by which anthocyanidins have hydrophobic properties and steroid nuclei which can mimic the androgens. The mimicry may also result in the binding of anthocyanidins to the steroid receptor, and at saturation, androgens (testosterone) are retained in the blood circulation (Omotuyi *et al.,* 2010). This is a new insight that *H. sabdariffa* extract possesses aphrodisiac properties and is in full agreement with previous studies by Ali *et al.* (2012) and Islam *et al.* (2019).

In this present study, rats exposed to AEHS exhibited a variance in sperm counts that resembles the trends shown by the testicular weight and testosterone levels. The medium-dose (500 mg/kg) group showed significantly lower sperm count when compared to all treatment groups. Spermatogenesis, a crucial process that entails transforming undifferentiated gametic cells into highly differentiated adult spermatozoa, typically takes 52 to 54 days in mature male rats (Liu *et al*., 2017). This study was able to evaluate the effects of AEHS therapy since the overall duration of dosing was at least 60 days. A previous study reported that *H. sabdariffa* calyx extract exhibited a protective role against streptozotocin-induced sperm damage in diabetic rats (Idris, 2012). In addition, another study delineated that the flower extract of *H. sabdariffa* was shown to increase sperm counts and decrease defective sperms in mice (Beheshti *et al.,* 2018). Nevertheless, the exact causes of the lowest sperm count in the medium dose (500 mg/kg) group of this study could not be determined due to a lack of evidence both from *H. sabdariffa* and animal experiments.

As for the medium-dose group, the rats in this group showed inconsistent data from body weight to sperm count parameters when compared to other treatment groups. It is possible to relate that this might be due to the indecisive confounding factors possibly individual variations of animals in the group instead of *H. sabdariffa* itself.

5. Conclusion

The present study has successfully provided information on the effects of AEHS on the male reproductive system in SD rats. Generally, the findings of this study demonstrated that the no-observed-adverse-effect level (NOAEL) of AEHS was less than 250 mg/kg/day. Thus, the current data suggest that AEHS should be consumed with caution particularly when the daily dose exceeds 250 mg/kg of body weight. It is also pertinent that future studies are highly required to further delineate the dose range effects, specifically on the aphrodisiac capability and to obtain an effective dose of AEHS.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by the Ministry of Agriculture's Herbal Research Grant Scheme (HRGS) (NH1015S020). The author would like to thank the Animal Research and Service Centre (ARASC) and School of Health Sciences, Universiti Sains Malaysia Health Campus, Kelantan, Malaysia for providing all the necessary facilities and assistance to conduct this study.

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