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Original Research Article

Hibiscus sabdariffa shows the potential of down regulating the expression of the Kiss1 gene in the ovary of Wistar rats

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Abstract

Objectives: This study investigated the Kiss1 gene expression in the ovary, following the administration of Methanolic extract of *Hibiscus sabdariffa* (MEHS) in Wistar rats.
Methods: Fifteen (15) rats with an average weight of 148 g were randomly divided into three (3) groups (n=5), A-C. Group A was given no treatment and served as the normal control group. Groups B and C received oral administration of 200mg/kg and 400mg/kg of MEHS respectively. The extract was administered once a day for 21 days.
Result: No significant difference was observed in the relative ovarian weight, reproductive hormone levels, and ovarian antioxidant levels in all the treatment groups compared to the control ($p>0.05$). There is a significantly lower intensity of expression of the Kiss1 gene in the ovarian tissue of group C ($p=0.011$) when compared to the control group. There is no significant correlation between the relative intensity of Kiss1 gene expression in the ovary and the reproductive hormones of all the experimental groups (p =0.879, 0.534, 0.133; r=0.081, 0.322, -0.686). MEHS caused no histopathological changes in the ovary at both treatment doses (200mg/kg and 400mg/kg).
Conclusion: MEHS shows the potential of downregulating the expression of the Kiss1 gene in the ovary, albeit at a higher dose. However, this effect lacks a regulatory mechanism on the reproductive hormones and ovarian antioxidative levels.
Keywords: *Hibiscus sabdariffa*, roselle, ovary, Kiss1 gene, gene expression, hormones, antioxidants, histology.

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1. Introduction

Plants are important sources of medicine - plants and plant products have been extensively used throughout history to combat and prevent diseases with varying degrees of success [1]. *Hibiscus sabdariffa* (HS) is an annual shrub that is thought to be native to tropical Africa, although it is also present in North America and Asia. HS is one of the most common flower plants grown worldwide [2], and has been used in folk medicine to treat a diverse spectrum of ailments including abscesses, cancer, cough, diabetes, fever, heart ailments [3], hypertension, and hyperlipidemia [4]. Clinical trials, as well as the pharmacological assessment of HS, have revealed anti-oxidant, anti-diabetic, anti-bacterial, nephroprotective and hepatoprotective effects [5]. HS contains numerous active phytochemicals and nutrients including alkaloids, tannins, saponnins, glycosides, phenols and flavonoids [6], proteins, oil and dietary fiber, lysine, arginine, leucine, phenylalanine, and glutamic acid [2].

Kiss1 gene is a protein-coding gene originally described as a cancer suppressor gene [7]. While the Kiss1 gene and its receptor, Kiss1r have been well recognized for their role in the inhibition of cancer metastasis [8], they are involved in other biological roles, particularly in the reproductive physiology of humans and other species [9]. Kisspeptin, a protein product of Kiss1 gene, is a key stimulatory agent for the release of gonadotrophin-releasing hormone (GnRH), and mediate the effects of sex steroid and metabolic cue on GnRH secretion [10]. While Kiss1/Kiss1r is predominantly expressed in the arcuate nucleus (ARC) and the anteroventral periventricular (AVPV) nucleus of the hypothalamus [11], it has also been shown to be well expressed in other tissues such as the ovary [12], pituitary gland, fat [13], testis [14], liver [15], and amygdala [16]. Inactivation or mutation of Kiss1r results in isolated hypogonadotropic hypogonadism, a disease characterized by an absent or incomplete sexual maturation, while a recent unique Kiss1r-activating mutation has been described to be involved in precocious puberty [10, 17]. Aside from the effect of the kiss1 gene on cancer biology and reproductive endocrinology, Kiss1/Kiss1r has also been implicated in several biological processes such as aging, vasoconstriction, adipocyte physiology, and metabolism [18]. The effect of kiss1/kiss1r expression on the hypothalamic-pituitary-gonadal axis and the consequent reproductive modulation has sparked interest in the effects of the kiss1 gene on other levels of the gonadal function. Previous studies have reported on the possible putative role of ovary-derived kiss1 gene in the regulation of follicular development, ovulation, steroidogenesis, and oocyte maturation [19], with ovarian kisspeptin shown to directly stimulate progesterone secretion [20]. Given the complex effect and reproductive potential of peripherally expressed kisspeptin, there is a need to investigate and isolate exogenous or endogenous substances or factors that may alter the level of kiss1 gene expression both on central and peripheral tissues. This present study investigated the level of Kiss1 gene expression in the ovary of adult Wistar rats, following the administration of MEHS.

2. Materials and Methods

2.1 Study setting

This experimental study was carried out in the research laboratory of the Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Science, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, and lasted for about 3 months.

2.2 Plant collection, identification, and extraction

The dried aerial part of HS was procured from the local market at Nnewi, Anambra state. The botanical identification and authentication were carried out in the Department of Pharmacognosy and Traditional Medicine, College of Pharmacy, Nnamdi Azikiwe University, Agulu Campus, Anambra State, Nigeria with identification number PCG/1474/A/031. The plant calyces were shade-dried and ground. 1000g of powdered plant sample was used for methanolic extraction as described by Okafor et al. [21]. The filtrate (extract) was then stored in the refrigerator at 4ºC. The extract was brought to solution at varying doses per ml on each day of administration and given according to body weight and group treatment doses.

2.3Animal procurement, Care and Handling

Fifteen (15) female Wistar rats were procured from the animal house of College of Health Sciences, Nnamdi Azikiwe University, Okofia Nnewi Campus and acclimatized for two (2) weeks (to exclude any intercurrent infection) under standard housing condition (ventilated room with o-12/12-hour light/dark cycle at 24 ± 2^0C). The rats were fed ad libitum with water and standard rat chow throughout the experimental period. Animal health status was monitored throughout the experiment according to the federation of European Laboratory Animal Science Associations (FELASA) guidelines.

2.4 Experimental design

Fifteen (15) rats with an average weight of 148 g were randomly divided into three (3) groups (n=5), A-C. Group A was given no treatment and served as the normal control group. Groups B and C received oral administration of 200mg/kg and 400mg/kg of MEHS respectively. The extract was administered once a day for 21 days.

2.5 Animal Sacrifice and Sample Collection

The animals were fasted overnight on the last day of MEHS administration and anesthetized using chloroform. 2 ml of blood each were collected from the animals by ocular puncture using capillary tubes into a plain tube for hormonal assay. The animals were sacrificed after blood collection, and the ovarian tissues were harvested, weighed, and divided into three parts. One was fixed in a 10% formal saline for histological processing and analysis. The second part was homogenized and used for oxidative status analysis. The last part was stored in an RNA protector-containing plain tube before gene analysis.

2.6 Serum Hormonal Assay

The blood was allowed to clot and centrifuged at 5,000rpm for 10 minutes within one hour after collection. The serum was extracted and used for the hormonal assay. AccuBind enzyme-linked immunoabsorbent assay (ELISA) microwells for Estradiol (EST), Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) purchased from Calibiotech Inc. (catalog number: E5380s), Bioassay technology laboratory China (catalog number: EO182Ra), Bioassay technology laboratory (catalog number EO179Ra) respectively. All analyses were carried out following the accompanying ELISA kit protocol for each parameter.

2.7 Biochemical Analysis of Oxidation

Superoxide Dismutase (SOD), Glutathione (GSH) and Catalase (CAT) were quantified in the ovary to determine the antioxidant status, using the ovarian tissue homogenate as described in our earlier studies (Okafor and Gbotolorun, 2018) [22].

2.8 Kiss1 RNA Extraction for ovary

Total RNA was extracted using the Zymo Research (ZR) Quick-RNA MiniPrep kit according to ZR specification. The homogenized samples in RNA protector were brought to room temperature (20-30 ºC). Then, 1 volume of RNA Lysis Buffer (1:1) was added and mixed. The mixture was incubated for 5 minutes at 25 °C and centrifuge at ≥ 12,000 × g for 1 minute. The supernatant was removed. A 600 µl volume of RNA buffer was added to the cell pellet and mixed properly. The resultant mixture was transferred into the Zymo-Spin IIIC column in a collection tube and centrifuged at ≥ 12,000 × g for 2 minutes. The column was transferred into a new collection tube. A 400 µl volume of RNA pre-wash buffer was added to the column and centrifuged at ≥ 12,000 × g for 30 seconds. The column was transferred into an RNase-free tube. 100 µl RNA recovery buffer was added to the Zymo spin IIIC column and centrifuged at ≥ 12,000 × g for 30 seconds. A 100 µl volume ethanol (100%) was added to the flow-through in the RNase free tube and mixed by pipetting. The mixture was transferred into the Zymo spin IC column in a collection tube and centrifuged at ≥ 12,000 × g for 30 seconds. A 400 µl volume of the RNA prep buffer was added to the column and centrifuged at ≥ 12,000 × g for 1 minute, the flow-through was discarded. An 800 µl volume of the RNA wash buffer was added to the column and centrifuged at ≥ 12,000 × g for 1 minute; the flow-through was discarded. The wash step was repeated with 400 µl volume of RNA wash buffer. The Zymo-spin IC column was centrifuged in an empty collection tube at ≥ 12,000 × g for 2 minutes. It was then transferred into an RNase-free tube. Total RNA was eluted by added 80 µl volume of DNase/RNase free water directly to the column matrix and centrifuged at 10,000 × g for 30 seconds. A 70 µl volume of the Total RNA extracted was transferred into an RNA stable tube supplied by Biomatrica (catalog number 93221-001) for storage of Total RNA at room temperature while 10 µl was used for quality control check on the Total RNA extracted.

2.8.1 RNA Detection

One gram of agarose powder was weighed and poured into 100 ml of Tris EDTA buffer in a Pyrex conical flask. It was heated using a microwave at 100°C for 5minutes. It was allowed to cool to 56°C and 6 µl volume of ethidium bromide was added to 100 ml of the gel mixture.

The gel was poured into the electrophoresis chamber and allowed to solidify. A 3 µl volume of loading dye was added to 7 µl volume of the Total RNA from each sample, the molecular marker was loaded in the first lane, followed by the samples. Electrophoresis was performed at 90 volts for 30 minutes. The gel was removed and viewed on the UV transilluminator; the picture of the gel was taken.

2.8.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The extracted total RNA was retro-transcribed and amplified using One Taq one-Step RT-PCR kit (catalog number NEB E5315S) by New England BioLabs incorporation according to the manufacturer's specification. Selected primers were used to target lymphocyte genes using MJ research Peltier thermal cycler polymerase chain reaction machine. The PCR was performed in a 50 µl volume reaction mixture containing 25 µl volume of one Taq one-step reaction master mix (2x), 2 µl volume of One Taq one-step enzyme mix (2x), 2 µl volume of each gene-specific forward primer (10 µM), 2 µl volume of each gene-specific reverse primer (10 µM), 9 µl volume of nuclease-free water and 10 µl volume of the RNA template was added. Negative control samples for the RT-PCR consisted of a mixture to which all reagents added except RNA. The PCR was started immediately as follows: Reverse transcriptase at 48°C for 30 seconds, initial denaturation at 94°C for 1 minute, denaturation at 94°C for 15 seconds, annealing at Tm - 5°C (the lowest melting temperature of each set of Kiss1 gene) for 30 seconds, extension at 68°C for 1 minute, denaturation step for 39 cycles, final extension at 68°C for 5 minutes and final holding at 4°C, forever. The Kiss1 gene nucleotide sequence (5'- 3') for the primers are as follows: forward primer - CTACGACTCCTTGTTGCTTTG, and reverse primer - TGATCTTCACTGTAGTTGGTGG.

2.8.3 Electrophoresis

5 µl of the amplified PCR products and DNA ladder were analyzed on 1% agarose gel containing ethidium bromide in 1X Tris EDTA buffer. One percent agarose gel was prepared by dissolving 1.0 g of LE Agarose powder in 100 ml volume of Tris Borate EDTA Buffer. The mixture was then heated in a microwave at 1000 C for 5 minutes, and allowed to cool to 56 °C and 6 µl volume of ethidium bromide was added to it. The agarose gel was poured into the electrophoresis chambers with gel comb, and allowed to solidify. Electrophoresis was performed at 90 volts for 30 minutes with the Edvotek tetra source electrophoresis machine, Bethesda, USA. After electrophoresis, the Kiss1 gene was visualized with the Wealtec Dolphin-Doc UV transilluminator and photographed.

2.8.4 Kiss1 Gene Relative Intensity of Expression

ImageJ 1.53a software was used to calculate the absolute intensity of expression from the generated gel images across all the experimental groups in the ovary. ImageJ generates the absolute intensity (derived by mean value multiplied by the pixel value or percent for each band) of each band. The absolute intensity is an integrated measure of the intensity and size of the band. The relative intensity was calculated by dividing the absolute intensity of each sample band by the absolute intensity of the standard.

2.9 Tissue processing

The harvested ovarian tissue samples were trimmed down to a size of about 3mm x 3mm thick for an easy study of sections under the microscope and fixed in 10% formalin. After fixation, dehydration of the fixed tissues was done in ascending grades of alcohol 50%, 70%, 95%, and 100%, and cleared in xylene. Staining was done with hematoxylin and eosin (H&E) and mounted using DPX, after which, the sections were viewed under the light microscope. Photomicrographs of these sections were obtained using the Leica DM 750 digital microscope computer software.

2.10 Statistical analysis

The data were analyzed using IBM statistical package for social science (SPSS) for Windows, version 23 (IBM Corporation, Armonk, New York, USA). One-way analysis of variance (ANOVA), post hoc LSD, student's t-test and Pearson's correlation analysis were used to test for significance in changes seen in the variables across groups. Tables and figures were used for the representation of data, and values were considered significant at $p<0.05$.

2.11 Ethical Statement

This study was approved by the Research Ethics Committee of Anatomy Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus. The experimental procedures of this study complied with ARRIVE guidelines, National Institutes of Health (NIH) guidelines, and National Health Research ethics committee of Nigeria (NHREC) guidelines for the care and use of laboratory animals. Animal health status was monitored throughout the experiment according to the federation of European Laboratory Animal Science Associations (FELASA) guidelines. No informed consent was required for this study.

3. Results and analysis

3.1 The effect of MEHS on the bodyweight.

Group A showed a significant increase (p=0.022) in body weight when the pre-administration weight was compared to the post-administration weight, while animals in groups B and C showed no difference in the pre and post-administration body weight (p=0.058, 0.089).

Table 1: The effect of MEHS on the bodyweight of female Wistar rats

Groups		Mean±SD	*p*-value
A (control)	Pre-administration	138.00±4.47	*0.022
	Post-administration	158.00±10.95	
B (MEHS 200)	Pre-administration	142.50±5.00	0.058
	Post-administration	157.50±9.57	
C (MEHS 400)	Pre-administration	162.00±10.95	0.089
	Post-administration	172.00±17.89	

Data were analyzed using the students' dependent t-test. Values were expressed as mean ± Standard deviation, and data were considered significant at $^{*}p<0.05$.

3.2 The effect of MEHS on the relative ovarian weight.

No significant change was observed in the relative ovarian weight when the treatment groups were compared to the control ($p<0.05$).

Table 2: The effect of MEHS on the relative ovarian weight of Wistar rats

Groups	Mean±SD	*p*-value
A (control)	0.050±0.017	0.230
B (MEHS 200)	0.033±0.008	
C (MEHS 400)	0.050±0.025	

Data were analyzed using One-way Analysis of Variance (ANOVA). Values were expressed as mean ± Standard deviation, and data were considered significant at $^{*}p<0.05$.

3.3 The effect of MEHS on the ovarian antioxidant levels.

There is no significant difference in the SOD, GSH, and CAT levels across the MEHS-treated groups when compared to the control group.

Table 3: The effect of the MEHS on the ovarian antioxidant levels in Wistar rats

	Groups	Mean±SD	*p*-value
SOD	A (control)	8.80±5.79	0.452
	B (MEHS 200)	12.00±0.28	
	C (MEHS 400)	13.65±1.06	
GSH	A (control)	14.79±5.13	0.362
	B (MEHS 200)	8.73±2.12	
	C (MEHS 400)	12.97±2.99	
CAT	A (control)	82.96±11.48	0.076
	B (MEHS 200)	54.79±8.15	
	C (MEHS 400)	79.19±2.39	

Data were analyzed using One-way Analysis of Variance (ANOVA). Values were expressed as mean ± Standard deviation, and data were considered significant at $^{*}p<0.05$.

3.4 The effect of MEHS on the reproductive hormonal levels

The serum LH, FSH, and EST levels across all MEHS-treated groups were not significantly different when compared to the control (p>0.05).

Table 4: The effect of the MEHS on the reproductive hormonal levels of Wistar rats

	Groups	**Mean±SD**	**P-value**
LH (mlU/ml)	A (control)	4.53±0.42	0.818
	B (MEHS 200)	4.53±0.71	
	C (MEHS 400)	4.34±0.41	
FSH (mlU/ml)	A (control)	6.03±0.58	0.323
	B (MEHS 200)	6.50±0.52	
	C (MEHS 400)	5.50±1.16	
Estradiol(pg/ml)	A (control)	20.80±3.11	0.627
	B (MEHS 200)	22.00±3.00	
	C (MEHS 400)	22.67±3.20	

Data were analyzed using One-way Analysis of Variance (ANOVA). Values were expressed as mean ± Standard deviation, and data were considered significant at $^{*}p<0.05$.

3.5 Correlation between the relative intensity of Kiss1 gene expression and serum hormonal levels.

There is no significant correlation between the relative intensity of Kiss1 expression variables and the reproductive hormones ($p>0.05$)

Table 5: Correlation between the relative intensity of Kiss1 gene expression and serum hormonal levels

		RIO
LH	Pearson Correlation	0.081
	Sig. (2-tailed)	0.879
FSH	Pearson Correlation	0.322
	Sig. (2-tailed)	0.534
EST	Pearson Correlation	-0.686
	Sig. (2-tailed)	0.133

RIO: Relative expression intensity in the ovary; LH: luteinizing hormone; FSH: Follicle stimulating hormone; EST: Estradiol; Values are significant at $p<0.05$.

3.6 Histopathological findings, PCR band images and relative expression intensity of kiss1 gene.

Plate A-C shows the histological section of rat ovary at different doses of MEHS. Plate 1 (group A) photomicrograph shows the histological section of a rat ovary administered only distilled water for 21 days. Ovarian follicles at varying stages of development, and the corpora lutea (indicating ovulation) can be seen. Plate 2 (group B) represents the histological section of rat ovary administered 400 mg/kg of MEHS for 21 days. The section shows follicles at varying stages of development, and the corpora lutea (indicating ovulation). Plate 3 (group C) represents the histological section of rat ovary administered 200 mg/kg of MEHS for 21 days. The section shows normal tissue histology with follicles at varying stages of development. Staining was done with H&E and photomicrography was taken at x200.

Figure 1: Histopathology

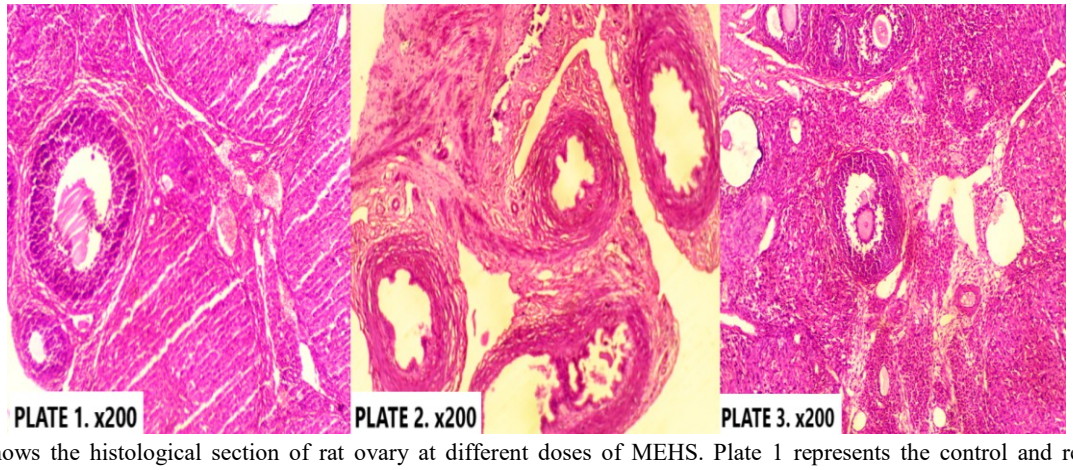
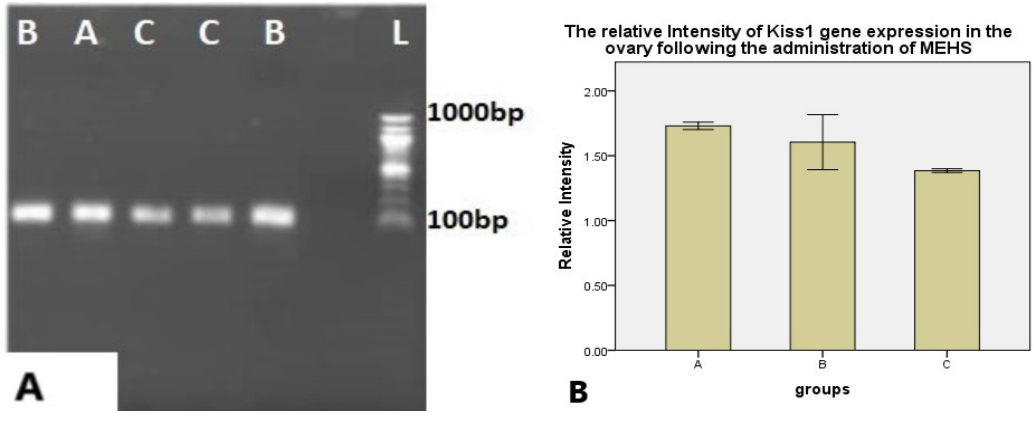


Plate A-C shows the histological section of rat ovary at different doses of MEHS. Plate 1 represents the control and received only distilled water. Plate 2 received 200 mg/kg MEHS, while plate 3 received 400 mg/kg MEHS.

Images labelled A & B shows PCR band images and the relative expression of Kiss1 gene in the ovary of Wistar rats in all the test groups, at different intensities. Figure **2A** represents RT-PCR result for Kiss1 gene expression on the ovary of Wistar rat analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. A, B and C are positive bands for the expressed KISS1 genes at 100bp while L is a 100bp-1000bp DNA ladder (molecular marker) sample. Figure **2B** represents the relative intensity of the Kiss1 gene expression in the ovary. There is a significant decrease in the ovarian Kiss1 gene expression levels in group C when compared to the control group ($p=0.011$) (Figure 2B). **A** represents group A; **B** represents group B; **C** represents group C.

Figure 2: PCR Band images



4. Discussion

Hibiscus sabdariffa has been used historically as a folk remedy for different diseases [23]. Although HS has been validated as an anti-obesity agent [24], there have been conflicting reports on its effect on body weight. Some studies reported a significant decrease in body weight following treatment with HS [24], others have reported a significant increase in body weight [25]. In this present study, treatment with 200 and 400 mg/kg MEHS caused no significant change in the body weight when the pre-administration body weight was compared to the post-administration body weight (p=0.058, 0.089). The bodyweight of the control animals increased significantly (p=0.022) when we compared pre and post-administration body weight (Table 1). Changes in the relative organ weight could be indicative of organ toxicity [26]. Our findings showed that MEHS did not cause any toxicities as no significant change was observed in the relative weight of the ovary when we compared the treated groups to the control group (Table 2).

Treatment with MEHS was able to alter the level of kiss1 gene expression in the ovary, in a dose-dependent manner. The result from this study showed that 400 mg/kg MEHS caused a significant decrease in the relative intensity of Kiss1 gene expression in the ovary (p=0.011) when compared to the control group; while 200 mg/kg caused no significant change (figure 2). Changes in the expression of the ovarian kiss1 gene are linked to various reproductive outcomes. Studies by Gaytán et al. [27] found that the selective suppression of ovarian kiss1 gene by COX-2 was able to disrupt the ovulatory process, suggesting a possible role of ovarian kiss1 gene in the control of ovulation. Also, studies by Gorkem et al.[28] reported an increase in the serum LH, testosterone (TT), and dehydroepiandrosterone sulfate in women with polycystic ovarian syndrome, with a positive and negative correlation observed between ovarian kisspeptin and the levels of TT and FSH. Interestingly, the observed decrease in the ovarian kiss1 expression in this present study did not show any effects on hormonal level as no significant changes were observed in FSH, LH and estradiol levels across treated groups, when compared to the control. The outcome of our study does not fit with the suggested action of the ovarian kiss1 gene on hormonal secretion and indicates a more complex mechanism behind the previously reported relationship of ovarian kisspeptin and reproductive hormones. Our study found no significant correlation between the relative intensity of Kiss1 expression variables and the reproductive hormones in this study (p>0.05) (Table 5).

Furthermore, previous studies on the reproductive effect of HS have also reported no significant changes in the serum LH, TT, estradiol and FSH [23, 29], and our study corroborates these findings.

HS extract has been shown to demonstrate antioxidant activities [30], and inhibit the generation of reactive radicals [31]. However, based on our findings, treatment with MEHS did not cause any significant change in the levels of SOD, CAT and GSH when compared to the control group (Table 3). Similarly, histological findings revealed no changes or abnormalities in the tissue architecture of the ovary (Figure 1).

Overall, MEHS was able to significantly decrease the expression of the kiss1 gene in the ovary but did not show any effect on the hormonal parameters, ovarian histoarchitecture, relative weight, or antioxidant status. While high dose MEHS was able to cause a downregulation of the expression of kiss1 in the ovary, it did not induce any reproductive hormonal or ovarian tissue toxicity, albeit for a short term consumption. Also, there is no evidence of any complementary effect of this downregulation on the reproductive hormones and ovarian antioxidative levels.

5. Conclusion

MEHS shows the potential of downregulating the expression of the Kiss1 gene in the ovary, albeit at a higher dose. However, this effect lacks a regulatory mechanism on the reproductive hormones and ovarian antioxidative levels. Further research needs to be carried out to understand the mechanism behind this effect and to isolate active phytochemicals from HS that may be responsible for altering the expression of the Kiss1 gene in the ovary.

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Conflict of Interest

The authors have no conflict of interest to declare

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