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Original Research Article

High Dose of *Hibiscus sabdariffa* L. Extract Affects Fetal Weight and Placental IGF-1 Receptor Expression in Feed-Restricted Maternal Wistar Rats

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ABSTRACT

Pregnancy-related undernutrition can alter Insulin-like growth factor 1 (IGF-1) receptor signaling, impacting fetal development. Research suggests that *Hibiscus Sabdariffa* Linn (*HSL*) extract consumption during pregnancy may influence fetal outcomes. However, limited studies have examined the effects of *HSL* on fetal growth through the IGF-1 receptor pathway in feed-restricted mothers. This study aims to investigate the impact of high doses of *HSL* extract on fetal weight in feed-restricted maternal rats by targeting the placental IGF-1 receptor pathway. Twenty *Wistar* rats were randomly divided into normal control, feed-restricted groups (FR), and FR groups treated with 100 and 200 mg/kg BW HSL extract. A Caesarean section was performed on day 18 of pregnancy to deliver the fetuses. The fetal weight was measured, and placental IGF-1 receptor expression was analyzed using RT-qPCR. The results revealed that fetal weight in FR group (833 ± 206.35) and FR+HSL groups (709 ± 235.93; 1453 ± 76.45) respectively, were significantly lower than the control group (1410 ± 76.75; p <0.05), and placental IGF-1 receptor expression in FR group (1.006 ± 0.116) and FR+HSL groups (1.127 ± 0.373; 0.847 ± 0.315) were significantly lower than the control group (1.658 ± 0.153) (p <0.05). Furthermore, placental IGF-1 receptor expression was positively correlated with fetal weight (r = 0.731, p < 0.05). In conclusion, the administration of high doses of HSL extract to feed-restricted maternal rats did not result in a significant increase in fetal body weight through IGF-1 receptor expression. Caution should be exercised when consuming high-dose *HSL* during pregnancy.

Keywords: Fetal weight, Food restriction, *Hibiscus Sabdariffa* L., Insulin-like growth factor 1 receptor.

Introduction

The nutritional status of the mother significantly affects fetal development and growth. The placenta is essential for integrating maternal nutritional indicators, such as low insulin and IGF levels, which can impact fetal growth and nutrient transfer. Maintaining fetal health during maternal undernourishment relies on this integration[1]. Limiting food intake during pregnancy may affect the expression of IGF-1 receptors in the fetal liver, potentially impacting the IGF system essential for fetal growth and survival[2]. Insulin-like Growth Factor 1 (IGF-1) and 2 (IGF-2) are crucial for fetal growth and development, with the IGF-1 receptor facilitating their action[3].

The IGF-1 receptor determines nutrient allocation between the mother and fetus, with the placenta facilitating essential nutrient transfer[4]. IGF-1 receptor signaling is crucial for placental angiogenesis, ensuring adequate oxygen and nutrient supply to the fetus. The activation of the receptor affects maternal vascular remodeling, which improves placenta perfusion[5].

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In wild-type mice, birth weight is reduced by 60% in cases of heterozygous deletion of the IGF-2 gene, which binds to the IGF-1 receptor.[6] Thus, deletion or malfunctioning of the IGF-1 receptor in animal models leads to fetal growth restriction (FGR) and decreased placental size, emphasizing its role in normal fetal development[7]. Placental nutrient transport capacity significantly influences fetal growth and development, with external factors, such as maternal smoking and alcohol consumption, reducing IGF-1 and IGF-1 receptor expression and birth weights[8]. Insulin resistance, which occurs when cells become less responsive to insulin, is associated with conditions like maternal diabetes and can influence IGF-1 receptor expression and activity, resulting in impaired fetal growth and development[9]. There is currently no proven therapy for impaired fetal growth related to maternal nutritional status. *Hibiscus sabdariffa* Linn (*HSL*) is a member of the *Malvaceae* family that can be found around the world in subtropical and tropical climates. *Hibiscus sabdariffa* Linn is a plant rich in bioactive compounds, including flavonoids, polyphenols, and tocopherols, as well as various organic acids, like oxalic, malic, and shikimic acid[10]. HSL has various health benefits, including treating abscesses, coughs, weakness, scurvy, fever, antihypertensive, antiseptic, diuretic, laxative, as well as having antioxidant[11], hepatoprotective, antihyperlipidemic[12], and cytotoxic properties[13]. The recent study explained that HSL has been observed to possess a high nutritional potential, particularly in the leaves, calyces, and seeds. The roselle calyx, which is utilized in the preparation of a range of beverages, has been documented to contain a substantial quantity of vitamins, minerals, flavonoids, protein, lipids, carbohydrates, and other nutrients[13,14]. However, its impact on maternal-fetal nutrition is less explored.

Studies suggest that HSL may influence fetal development by modulating reproductive hormones and IGF-1 levels. Administration of

250 mg/kg oral doses of aqueous extract of *HSL* calyx caused significantly reduced levels of follicle-stimulating hormone (FSH), testosterone, luteinizing hormone, and prolactin in male Wistar rats[15]. The antioxidant properties of *HSL* can enhance cellular health and function, which is crucial for maintaining normal IGF signaling pathways [16]. *HSL* has been found to increase hemoglobin levels in pregnant mothers, which is important for fetal development and maternal health[17]. Consumption of *HSL* extract during pregnancy has been linked to increased birth weights, attributed to its effects on serum glutamate pyruvate transaminase (SGPT) levels[18], and improved maternal health.

While studies have shown the potential impact of *HSL* on maternal-fetal health, its influence on placental and fetal development under food restrictions is less explored. In this study, we propose a propose novel investigation into the potential of HSL to mitigate the negative effects of food restriction on fetal growth, specifically exploring its interaction with the placental IGF-1 receptor pathway. This study aims to investigate the effect of high doses of *HSL* calyx extract on fetal weight in maternal feed-restricted rat conditions through the IGF-1 receptor pathway in the placenta.

Materials and Methods

Hibiscus sabdariffa L. *(HSL) extraction*

Mature calyces of *HSL* were purchased from the Tropical Biopharmaceutical Study Center at Bogor Institute of Agriculture (IPB University) in Indonesia. The extraction process was conducted at the Balitro laboratory of the Indonesian Ministry of Agriculture using a maceration technique with methanol as the solvent. A 2000-gram *HSL* calyx powder was extracted using a maceration method in methanol solution for 48 hours, with agitation occurring intermittently. Powder extraction was performed via repeated rinsing with a fresh methanol solution. The filtrate was evaporated at 40°C using a vacuum rotary evaporator (Heidolph Hei-VAP, Germany) to recover methanol powder. The concentrated extract was transferred into a sterile beaker, sealed with aluminum foil, and then refrigerated.

The *HSL* extract was then assessed for its water content to determine the appropriate dosage. The analysis revealed that the *HSL* extract contained 25.14% water, necessitating adjusting the dosage calculation to account for the water content. To ensure the safety of the extract for consumption, it was evaluated based on the guidelines provided by the National Food and Drug Authority [19] (see Table 1).

The water content and ash content were determined using the Gravimetric method as follows:

Water content: approximately 2 grams of extract were placed in a pre-calibrated porcelain cup and dried in an oven at 105°C for 1 hour, desiccation for about 15 minutes. The cup was weighed repeatedly until a stable weight was obtained, indicated by a difference of no more than 0.5 mg or 0.25% between consecutive weighings.

Total Ash Content: 2 grams of the extract were placed in a platinum or silicate crucible, ignited at 600°C, cooled, and weighed until a constant weight was achieved. Charcoal residue, if present, was not removed; instead, hot water was added, filtered through ash-free paper, and incinerated. The remaining ash and filter paper were incarnated until a constant weight was reached, then weighed to calculate the ash content relative to the air-dried material.

Mold/Yeast Count: 1 mL of sample from dilutions ranging from 10^{-2} to 10^{-5} was transfered to sterile petri dishes using a separate sterile pipette. Each dilution was plated on Potato Dextrose Agar (PDA) media, mixed well, solidified, and incubated at 25°C for 5 days in an inverted position.

Pb Levels Analysis: The Pb levels were determined using Atomic Absorption Spectroscopy (AAS) (Spektrometer AA PinAAcle 900T; USA). The instrument was optimized, and reductants were prepared, including a solution of 0.5% NaOH + 0.2 g NaBh4 to reduce non-target compounds chemically. A 3% HCl protective solution was used during the analysis process.

Animals and Treatment

This study used 20 female *Wistar* rats, aged 2 – 3 months, purchased from PT Bio Farma Bandung, Indonesia. After 7 days of acclimatization, the female rats were mated with five males alternately. Before mating, the estrus cycle was observed using a vaginal swab technique stained with Giemsa. Female rats in the proestrus and estrus phases were considered ready to mate. Male mice were placed in female rats' cages in a 1:1 ratio. Mating was considered successful if the vaginal swab showed vaginal debris or spermatozoa could be found under microscope observation, and this was determined as the first day of pregnancy.

Pregnancy preparation was then carried out, and food restriction was implemented by limiting food intake to 50% of the average amount consumed by regularly fed rats[20]. It was observed that the daily feed consumption ranged from 20 grams per head, so the calculated limit was 50% of this amount, approximately 10 grams/head/day. The standard rodent chow produced by PT Citra Ina Feedmill (Citrafeed) Indonesia contained a maximum of 12% water, a minimum of 20% protein, a maximum of 4% fat, a maximum of 4% coarse fiber, 12% Calcium, and 0.7% phosphorus.

On the first day of pregnancy, the twenty (20) rats were randomly assigned to four groups of five rats per group (one animal per cage). The experimental groups were categorized: Normal control groups, which consisted of animals receiving only water and standard rat chow in *ad libitum quantities*; feed-restricted (FR) groups, which received only water and a 50% restricted diet; FR groups with 100 mg/kg BW/day and 200 mg/kg BW/day.

The *HSL* extract was administered orally using an oropharyngeal cannula for 18 days of pregnancy. Dosage calculations were adjusted based on body weight at the start of pregnancy. Pregnancy termination was performed by cesarean section (SC) on the 18th day of pregnancy under anesthesia (xylazine and ketamine cocktail 0.1 ml/100-gram BW intraperitoneally). The placenta was removed after opening the abdomen and uterus. The placenta was weighed and stored in a tube in a refrigerator at -80 °C until used for analysis.

Fetal Weight Measurement

After a cesarean section, the weight of the fetus is measured using a digital scale calibrated in milligrams (Adam equipment). The fetus is weighed after being removed from the amniotic membrane.

RT-qPCR analysis

The placental tissues were homogenized using a sonicator and then mixed with 800 µl of TRizol®, TRI Reagent® (Zymo Research; China). The homogenate was centrifuged, and the supernatant was transferred to a new nuclease-free tube to remove particulate debris. Total RNA was isolated using the Direct-zol RNA Mini-Prep Plus Kit from Zymo Research following the manufacturer's protocol. RNA concentration is ± 602 ng/µL, taken based on a purity value of 1.8 – 2.0. Synthesis cDNA using PCR-SensiFAST SYBR No-ROX One-step kit 500 rxns (Bioline, Taunton, MA). All samples were measured in triplicate. RT-qPCR was performed in one step with 40 cycles on an Applied Biosystems 7500 Fast machine (Thermo Fisher Scientific, CA). The data were analyzed by relative quantification using $2^{-\Delta\Delta Ct}$ methods[21]. The primer sequences for the IGF-1 receptor and the endogenous control (glyceraldehyde phosphate dehydrogenase /GAPDH) are provided in Table 2.

Ethical Approval

All the procedures in this study were approved by the ethical committee of the Faculty of Medicine /DR. Cipto Mangunkusumo National Referral Hospital, Universitas Indonesia (No. KET-1436/UN2.F1/ETIK/PPM.00.02/2023).

Study Period and Location

The study was conducted at the Animal Research Facility (ARF) Laboratory, Indonesia Medical Education and Research Institute (IMERI) Faculty of Medicine Universitas Indonesia, to induce pregnancy in rats through feed restriction and extract intervention over six months. Sample preparation, storage, and qRT-PCR analysis were performed at the Biochemistry laboratory, Molecular Biology, and

Proteomic Core Facilities (MBPCF) at IMERI, Faculty of Medicine, Universitas Indonesia.

Statistical Analysis
Data analysis was conducted using Prism GraphPad Software. All data are presented as Mean ± S.E.M. One-way analysis of variance (ANOVA) was used for data analysis, with a significance level set at *p*-value < 0.05.

Table 1: HSL extract analysis results

Parameter	Result	Standard	Analysis Methods
Ash content	2.94	< 5.6 %	Gravimetric analysis
Mold/yeast	Negative	< $5x10^5$ coloni/g	Pour Plate Method
Pb	Not detected	< 10 mg/kg	Atomic absorption spectroscopy (AAS)

Table 2: Primer sequences

Genes	Forward primer	Reverse primer	Product (bp)	Annealing
IGF1-receptor	CAAAATGAGCGCACCTCCAA	CTTCAGCGGAGCACAGTACA	295	60°C.30s
GADPH	GAAGGTCGGTGTGAACGGAT	AACTTGCCGTGGGTAGAGTC	156	60°C.30s

Results and Discussion

The Effect of Hibiscus sabdariffa L. Extract on Fetal Weight
This study revealed that a 50% food restriction for 18 days during pregnancy significantly reduced fetal weight compared to the control group. Moreover, administering Hibiscus sabdariffa (*HSL)* extract at doses of 100 and 200 mg/kg BW/day for 18 days during pregnancy also led to a significant decrease in fetal weight compared to the control group (**Figure 1**). Although fetal weight tended to be lower compared to the group subjected to the 50% food restriction alone, the difference was not statistically significant.

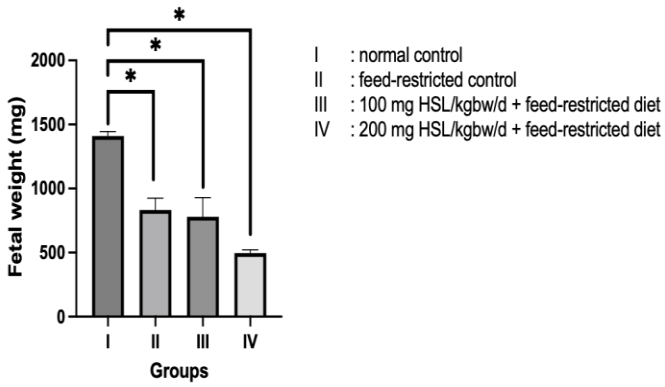

Figure 1: The impact of Hibiscus sabdariffa L. extract on fetal weight in each group. **p<0.05* vs. control.

Research has demonstrated that maternal undernutrition during gestation can induce intrauterine growth restriction (IUGR). These findings are consistent with a study by Zeng He et al., which found lower fetal body weight in the Prenatal Food Restriction (PFR) group compared to the control group. Maternal and fetal serum corticosterone levels were significantly elevated in the prenatal feed restriction group, and the expression of the adrenal IGF-1 signaling pathway, including IGF-1, IGF-1 receptor, and Akt1, was suppressed[20]. Moreover, the fetal body and liver weight were also gradually decreased with decreasing maternal nutrition during late pregnancy in sheep[22].
Studies in rats have suggested that consuming *HSL* extract during pregnancy and lactation can affect postnatal weight gain, body mass index, and delayed organ development in offspring[23]. Research on rats has also indicated that consuming *HSL* aqueous extract during pregnancy can reduce litter sizes and increase litter birth weights, possibly through the gluconeogenic activities of elevated SGPT [24]. Our study results indicate that consuming *HSL* calyces extract in high doses did not improve fetal weight in the context of a maternal feed-restricted diet.

The Effect of Hibiscus sabdariffa L. Extract on IGF-1 Receptor
A 50% food restriction during pregnancy led to a decrease in placental IGF-1 receptor expression compared to the control group. Consumption of *HSL* during pregnancy did not result in increased IGF-1 receptor gene expression. Our findings indicated that IGF-1 receptor gene expressions in all treatment groups and the food restriction group were lower than in the normal control group (*p-value < 0.05*; refer to Figure 2).

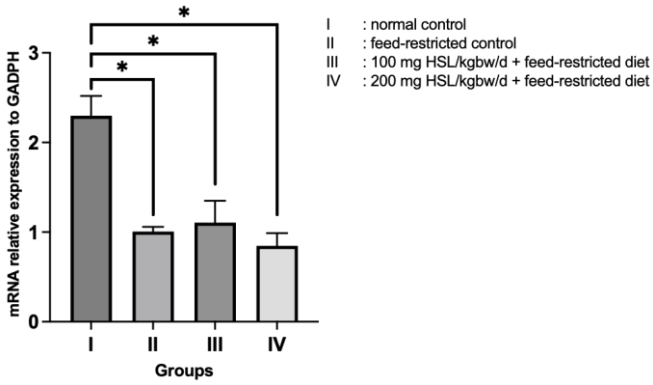

Figure 2: The impact of Hibiscus sabdariffa L. extract on placental IGF-1 receptor mRNA Expression. **p <0.05* vs. control.

The IGF-1 receptor binds to insulin-like growth factor-1 (IGF-I) and activates downstream signaling pathways that influence fetal growth and development[253]. It is mainly expressed on the maternal-facing plasma membrane of the trophoblast, the transporting and hormone-producing epithelium of the human placenta. The binding of insulin and IGF-1 to these receptors promotes metabolic and mitogenic processes, regulating placental and fetal development[26 27]. Placental and maternal IGFs play a crucial role in promoting placental growth and function, including amino acid transport. IGFs, particularly IGF-1, are essential

regulators of fetal development. The syncytiotrophoblast microvillous plasma membrane is rich in insulin and IGF-1 receptors, and their effects in the mother's blood and at the mother-fetal interface act as powerful stimulators of placental growth and function[28].

Maternal nutrient restriction (MNR) diets can alter placental microstructure and nutrient transport capacity, potentially affecting IGF-1 receptor expression. Studies have shown that MNR during early pregnancy in rabbits did not affect conception rates or embryo survival; however, it did alter the expression of IGF genes in the fetal liver, potentially impacting placental function and fetal growth[29]. Additionally, it also changes placental morphology, reduces placental weights, and alters gene expression profiles[2]. Research on mice has demonstrated that food restriction during early pregnancy can lead to changes in placental morphology and gene expression, including the IGF-1 receptor[30].

In our study, maternal feed restriction during pregnancy in rats resulted in decreased IGF-1 receptor gene expression in the placenta, which might be associated with the maternal IGF gene expression. Administration of *HSL* extract to the feed-restricted groups did not improve IGF-1 receptor gene expression. *Hibiscus Sabdariffa* L. calyx contains various beneficial compounds such as carotenoids, organic acids, anthocyanins, flavonoids, polyphenol acids, sugars, polysaccharides, and fatty acids. Flavonoids in *HSL*, including quercetin[10], have been shown to downregulate the phosphorylation statuses of the IGF-1 receptor and insulin receptor substrate 1 (IRS1), affecting the associated signaling pathway[31]. Quercetin treatment resulted in a reduction in IGF-1 receptor mRNA expression, downregulated p-Akt, p-ERK1/2, and anti-apoptotic protein expression, suggesting that quercetin can inhibit the development and progression of prostate cancer in *Sprague-Dawley* rats[32]. Therefore, caution should be exercised when using *HSL* extract during pregnancy as it may impact the signaling pathways related to the IGF-1 receptor.

Correlation Between Placental IGF-1 Receptor Expression and Fetal Weight

This study was analyzed to determine the correlation between placental IGF-1 receptor expression and fetal weight. The findings revealed a significant positive correlation between placental IGF-1 receptor expression and fetal body weight ($p<0.05$; Figure 3). The correlation coefficient (r) value of 0.731 indicates a strong correlation, suggesting that increased expression of the placental IGF-1 receptor is associated with higher fetal weight.

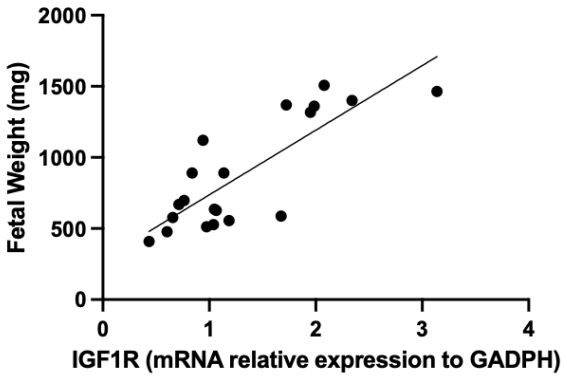

Figure 3: Correlation between placental IGF-1 receptor expression and fetal weight. *P-value < 0.05, r=0.731*

The IGF-1 receptor plays a crucial role in fetal growth and development, as it is activated by insulin-like growth factors (IGFs) such as IGF-1 and IGF-2, which are essential for cell proliferation and growth[25]. Dysfunction or absence of the IGF-1 receptor can lead to significant growth deficiencies, as seen in studies on mice lacking the IGF-1 receptor gene (Igf1r null-zygotes) that exhibit a 45% reduction in birth weight and do not survive beyond birth[6]. Our results underscore the importance of placental IGF-1 receptor expression in regulating fetal weight, highlighting its role in fetal growth.

Conclusion

In pregnant rats on a 50% feed-restricted diet, the extract of *Hibiscus sabdariffa L.* (HSL) calyces reduces fetal weight by influencing the placenta's expression of the IGF-1 receptor gene in a dose-dependent manner. Therefore, using high doses of HSL while pregnant should be done with caution. To completely comprehend the impact of HSL calyces extracts on placental processes, pregnancy outcomes, and their potential for translational studies in humans, more investigation is required.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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