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Phyllanthus muellerianus Kuntze exell (Euphorbiaceae) improves maternal reproductive outcomes, fetal growth, and modulates placental oxidative stress/inflammation in a rat model of intrauterine growth restriction

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Abstract

Background: Intrauterine growth restriction (IUGR) is associated with placental oxidative stress and inflammation, contributing to poor pregnancy outcomes. *Phyllanthus muellerianus* is a medicinal plant rich in bioactive compounds with reported antioxidant and anti-inflammatory effects. This study investigated the effects of *Phyllanthus muellerianus* extracts in a rat model of chronic restraint stress (CRS)-induced IUGR.

Methods: Thirty-five pregnant rats were exposed to CRS (6h/day) and treated from pregnancy days 7 to 18 with distilled water (10 ml/kg), aqueous, or methanolic extract (372 mg/kg). Maternal (resorption, post-implantation loss) and fetal outcomes (fetal weight, placenta efficiency), lipid profile (HDL, LDL, triglycerides), reproductive hormones (estradiol, progesterone), placental oxidative stress (malondialdehyde, superoxide dismutase, reduced glutathione), and inflammatory cytokines (TNF-α, IL-1β) were assessed.

Results: CRS induced significant alterations, including decreased maternal body weight (30%), mean live fetuses (40%), progesterone ($p< 0.05$), HDL (56%), fetal growth indices ($p< 0.05$) and superoxide dismutase activity (41%), alongside increased LDL (47%), malondialdehyde ($p< 0.01$) and pro-inflammatory cytokines ($p< 0.01$-0.001). Treatment with *P. muellerianus* significantly reversed these changes, demonstrating materno-protective, antioxidant, anti-inflammatory, and progesterone-enhancing effects. No adverse effects were observed in unstressed subjects.

Conclusion: *Phyllanthus muellerianus* improves maternal and fetal outcomes in CRS-induced IUGR, likely through modulation of oxidative stress, inflammatory pathways, and endocrine function. These findings support its potential as a therapeutic candidate for managing IUGR.

Keywords: Chronic stress; fetal growth; inflammation; oxidative stress; *Phyllanthus muellerianus*; placenta.

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Background

Intrauterine growth restriction (IUGR) is a pregnancy-related disease whereby the fetus fails to meet its growth potential in the maternal uterine environment [1]. This usually results in restricted fetal growth and the most commonly used diagnosis parameter of IUGR is fetal weight [2, 3]. About 1 out of 5 pregnancies experience IUGR worldwide, resulting to almost 30 million babies affected each year, thereby contributing to perinatal mortality and morbidity [4]. The etiology of IUGR is complex, but the onset is mostly due to inadequate fetal availability of nutrients and/or oxygen caused by maternal hormonal imbalance and/or placental dysfunction. Actually, the placenta is the organ that connects the fetus to the maternal uterine walls to enable exchange of nutrients and waste products [5]. However, factors such as inadequate maternal nutrition, hyperandrogenism and psychological stress can disrupt placental development and function during early pregnancy [6-8].

Chronic psychological stress (CPS) triggers hypercortisolism that suppresses the hypothalamic-pituitary-ovarian (HPO) axis and reduces ovarian steroidogenesis. Progesterone drop witnessed during CPS inhibits angiogenesis and proliferation of the placenta, thereby reducing fetal body weight, a hallmark of IUGR [9]. Nitric oxide (NO) is the main vasodilator agent in the placenta, facilitating fetal homeostasis and growth. Past studies showed that prenatal CPS results in higher risk for negative pregnancy outcomes including embryo resorption and reduced litter size [10]. Kaya et al., [11] demonstrated that prenatal immobility stress impairs fetal growth by changing the placental morphology and physiology. During organogenesis, maternal CPS can impair the placental barrier and the transplacental passage of cortisol may disturb basal metabolism in the developing fetus with long-lasting changes in future offspring [12]. Alteration of maternal lipid metabolism is shown to be implicated in various pregnancy complications, contributing to poor fetal growth [13, 14].

Oxidative stress and inflammation play a key role in the pathophysiology of IUGR by impairing placental function and development [11]. Hypercortisolism increases reactive oxygen species (ROS) and weakens the antioxidant defense, which can harm fetal growth [15]. Pro-inflammatory cytokines are regularly activated during oxidative damage of cellular processes [16]. To prevent IUGR, low-dose aspirin (81 mg/day) is a recommended treatment from 16 weeks of pregnancy until delivery [17, 18]. Diazepam, a synthetic anxiolytic and muscle relaxant, has been used to mitigate pregnancy-related complications [19]. However, its use is associated with potential risks, including teratogenicity and muscle fatigue [20].

Phyllanthus muellerianus (Euphorbiaceae) is an interesting plant widely used in African ethnomedicine for the management of reproductive disorders. Phytochemical investigations have revealed the presence of several bioactive compounds with antioxidant, anti-inflammatory and hormone-modulating activities [21-23]. Its consumption also appears to be safe during pregnancy and breastfeeding [24]. Previous work demonstrated the pro-sexual effects of this plant in stressed female rats [25]. Despite these beneficial roles in reproductive health, its impact on placental oxidative stress and fetal growth under chronic maternal stress remains unclear. Therefore, this study investigated the protective effects of *Phyllanthus muellerianus* on chronic restraint stress-induced intrauterine growth restriction in rats. We hypothesized that *Phyllanthus muellerianus* would prevent stress-induced IUGR by limiting placental oxidative stress and inflammation, thereby enhancing placental efficiency, fetal growth and pregnancy outcomes.

Methods

Ethical considerations

This study was conducted following institutional guidelines for the care and use of laboratory animals, as described by the international accepted standards for animal use of the European Economic Community guidelines; EEC. 2010 Council Directive 2010/63/EU of 22 November 2010. Approval of the experimental protocol was granted by the scientific committee of the Animal Biology Department, Faculty of Science, University of Dschang (Approval number: 1728CEI-UDS/22/07//2020).

Animals

Adult female virgin (3-4 months) and male (5-6 months) Wistar rats were obtained from the animal facilities of the Faculty of Sciences, University of Dschang. They were kept under room conditions of 12h light/dark cycle, temperature (23-25°C) and humidity (60±2%). All animals had free access to food and water.

Mating and pregnancy detection

Female rats were mated during ten consecutive days with vigorous male rats at the ratio of 3:1. Vaginal smears were collected each morning and placed on a clean slide for sperm cells check under the light microscope at 40X magnification. Sperm positive slides were considered pregnant, and the day of observation was noted as pregnancy day 1 (PD1). Pregnant rats received phytoestrogen-free (soybeans) food together with *ad libitum* access to water. To minimize potential confounders, pregnant rats were randomly assigned to groups, and all measurements were performed by a blinded operator.

Plant collection and extracts preparation

P. muellerianus (PM) fresh root barks were harvested in Tonga (West region of Cameroon) and authenticated under the voucher N° BWPV03 at the National Herbarium of Cameroon. These barks were separately shade-dried for a week, later transformed into powder and used for the preparation of aqueous and methanolic extracts. Aqueous extract (AE) of PM was obtained by infusing 250 g of powder in 1000 ml of distilled water for one hour. The infusion was filtered, oven-dried (45°C) for 2 days and 21.15 g of residue obtained (Extraction yield: 8.46%). The methanolic extract (ME) of PM was prepared after maceration of 250 g of powder in 1000 ml of methanol during 72 h at room temperature. The filtrate was collected and evaporated (using rotatory evaporator at 40°C) to collect 15.17 g of a brownish residue (Extraction yield: 6.07%) [25, 26].

Dose selection

The aqueous and methanolic extracts of PM were given at the dose of 372 mg/kg, which was the most effective dose in our preliminary study [25]. Diazepam was administered at the dose of 3 mg/kg [19].

Induction of Intrauterine growth restriction by chronic restraint stress model

Chronic Restraint Stress (CRS) also called immobilization stress was used to induce intrauterine growth restriction (IUGR) in pregnant rats following the protocol of [11]. For this purpose, a locally manufactured restrainer was constructed using an opaque plastic cylindrical tube (diameter 6.5 cm and length 21 cm). This tube was made of 6 holes (for ventilation) perforated woody cover on the left, and a scrollable plastic cover on the right. An additional opened plastic cylindrical tube was inserted into the main tube and attached by a metallic belt with an external screw to adjust the animal size and limit any movement. Each pregnant rat was inserted into this restrainer for 6 hours/daily as from PD7 to PD18 (start of organogenesis to full fetal development) without food or water.

Experimental groups and treatments

70 sperm-positive pregnant rats were randomly assigned into 7 groups (n= 10) as follows: Group 1 or normal control animals were unstressed pregnant rats receiving distilled water (10 mL/kg); group 2 or negative control made of CRS pregnant rats receiving distilled water (10 mL/kg); group 3 or positive control was made of CRS pregnant rats treated with diazepam (3 mg/kg); groups 4-5 were CRS pregnant rats treated with either aqueous or methanolic extract of *P. muellerianus* (372 mg/kg); groups 6-7 made of unstressed pregnant rats receiving aqueous or methanolic extract of *P. muellerianus* (372 mg/kg).

Plant extracts were administered by oral gavage while diazepam was given through intraperitoneal injection as from PD7. Body weight growth was calculated at the end of experiment (Figure 1A).

Necropsy and sample collection

On the 18th day of pregnancy, rats were anesthetized via the intraperitoneal injection of diazepam (10 mg/kg) for muscle relaxation, followed by ketamine (50 mg/kg) to inhibit consciousness and pain. After successful anesthesia, blood was collected from the abdominal artery into heparin tubes and centrifuged at 3000 r.p.m for 15 mins to collect plasma stored at -20°C until use. We confirmed the pregnancy of 5 rats per group at euthanasia. The gravid uterus was removed to determine pregnancy outcomes. Each fetus and placenta were dissected from the uterus, blotted on clean gauze and weighed separately. All fetuses were examined for any external malformations.

Maternal reproductive outcomes

Maternal reproductive outcomes were represented by the number of implantations (number of live fetuses + number of dead fetuses + number of resorptions), resorption of fetuses in the uterus (empty uterine nodules), live (reddish) and dead (dark or brown) fetuses. Post-implantation loss and fetal viability were calculated according to [27, 28]:

Post-implantation loss (PIL) **=** 100 × (Number of implants – Number of live fetus/Number of implantation sites);

Fetal viability **=** Number of live fetus **×** 100/Number of implants.

Fetal growth indices

For each fetus, the crown-rump length (CRL) was measured as the length from the tail to the head of each fetus and the mean CRL of each group recorded. Placenta efficiency was equally calculated using the formula:

Placenta efficiency**=** Fetus weight/Placenta weight [27].

Enzyme-linked immunosorbent (ELISA) assay

Corticosterone concentration was determined in plasma using the Mlbio (Good Elisa kit producers, China) ELISA kit. Plasma levels of estradiol and progesterone were measured using the Fortress Diagnostics ELISA kit (Ref: BXE0860A). Plasma interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were determined using the Sandwich ELISA method provided by R&D system (Cat: DY501 and Cat: DY510 respectively).

Lipid profile of maternal plasma

Plasma lipid profile including total cholesterol (TC), high density lipoprotein (HDL) and triglycerides (TG) was measured by colorimetric method using commercial kits (Dutch Diagnosis, Netherlands). Low density lipoprotein (LDL) levels were calculated from the Friedewald's formula: LDL-C **=** TC - HDL-C - (TG/5)[29]. The atherogenic index of plasma (AIP) was calculated as AIP **=** Log (TG/HDL-C) [30].

Determination of placenta oxidative stress markers and nitric oxide

Part of placentas were crushed in 10mM Tris buffer (pH= 7.4) and after cold centrifugation (5°C, 3000 rpm for 10mins), homogenates were stored in Eppendorf tubes at -20°C until analysis. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione reductase (GSH) and nitric oxide (NO) were assessed in placenta tissues. The lipid peroxidation marker MDA was monitored according to thiobarbituric acid reaction [31]. GSH level and SOD activity were assessed by using the protocols of Giustarini et al., [32] and Serra et al., [33] respectively. NO content was measured using Roche Diagnostic kit (Germany) based on colorimetric analysis at 540 nm.

Statistical analysis

All data are presented as mean±standard error on mean (SEM) of at least five independent subjects. Normality test was confirmed for all data using the Kolmogorov-Smirnov test. Differences between controls and intrauterine growth restricted groups were analyzed using one way analysis of variance (ANOVA) followed by Tukey post-hoc test to separate means. Mortality rate was expressed as a group proportion, and no statistical analysis was done. The percentage improvement was calculated in case of no statistical difference as [(mean of treated group-mean of stressed group)/mean of control group] **×** 100. All analysis and graphs were prepared using the software GraphPad Prism 8.4.2 (Boston, MA, United States). Statistical significance was set at $p< 0.05$.

Results

Effects of P. muellerianus on maternal body and reproductive organs weights

Figure 1B shows that maternal body weight growth was reduced in CRS pregnant dams (16.34±1.98; 30%) compared to the intact pregnant rats (23.28±2.83). Treatment with diazepam or plant extracts did not reverse body weight loss as compared to the CRS control. The relative weights of the ovary (Figure 1C) and uterus (Figure 1D) were also reduced in the CRS control by 14% and 33% respectively, compared to intact group. Similar to diazepam, plant extracts showed an improvement of the relative weights of ovary (AE372: 24%, ME372: 15%) and uterus (AE372: 46%, ME372: 64%) compared to the CRS control. The administration of plant extracts to intact pregnant rats did not affect the weights of reproductive organs.

Effects of P. muellerianus on maternal reproductive outcomes

During the experiment, a mortality rate of 22.2% (2 dams on 9) was recorded in pregnant rats exposed to CRS whereas no death occurred in the intact group (Table 1). Treatment with the methanolic extract reduced mortality rate (14.6%) compared to the CRS control. Administration of plant extracts to unstressed pregnant rats did not result in any mortality. Maternal reproductive outcomes registered on day 18 of pregnancy are presented in Table 1. The number of implants, mean live fetus and fetal viability, dropped by 30%, 40% and 17% respectively in the CRS pregnant rats compared to the intact group. Additionally, a 100% increase of resorptions and post-implantation loss was observed in the CRS pregnant group for in comparison to the intact group. Aqueous and methanolic extracts improved all pregnancy outcomes, including number of implants (AE372: 33%, ME372: 33%), number of resorptions (AE372: 50%, ME372: 100%), live fetuses (AE372: 38%, ME372: 48%), post-implantation loss (AE372: 29%, ME372: 100%) and fetal viability (AE372: 5%, AE372: 17%), compared to the intact group. Plant extracts administered to unstressed pregnant rats did not change reproductive outcomes compared to the intact control.

Effects of P. muellerianus on fetal growth indices

As presented in Figure 2, an important reduction of the fetal mean weight (Figure 2A, $p< 0.05$; 51%), placenta efficiency (Figure 2C, 63%), crown rump length (Figure 2D, $p< 0.05$; 30%) was noticed in the CRS group receiving DW as compared to the intact group. This effect was coupled to a non-significant rise of placenta mean weight (Figure 2B, 23%) compared to intact control. Just like diazepam, *P. muellerianus* especially the methanolic extract improved fetal growth indices as fetal weight (29%), placenta efficiency (71%) and CRL ($p< 0.05$; 26%) in comparison to CRS control. In addition, the administration of plant extracts relatively maintained fetal growth indices similar to the intact group, except the aqueous extract which reduced ($p< 0.05$) CRL.

Effects of P. muellerianus on maternal plasma corticosterone, 17β-estradiol and progesterone levels

CRS led to a 32% increase of corticosterone level compared to the intact control (Figure 3A). Conversely, a reduction of 17β-estradiol (Figure 3B, 13%) and progesterone (Figure 3C, $p< 0.05$) was found in CRS exposed pregnant dams. Diazepam significantly ($p< 0.05$) reversed corticosterone level, without plant extracts effect compared to the CRS group. Administration of plant extracts to CRS pregnant dams led to the restoration of reproductive hormones, with marked effect on progesterone level by the methanolic extract ($p< 0.01$) compared to the CRS control. Plant extracts treatments to unstressed pregnant dams improved 17β-estradiol ($p< 0.05$) and progesterone ($p< 0.001$) as compared to intact pregnant dams.

Effects of P. muellerianus on the plasma lipid profile

A reduction of HDL (Figure 4B, 56%), TC (Figure 4A, 13%) and atherogenic index (Figure 4E, 45%) was observed in the CRS group as compared to the intact group. Contrarily, LDL (Figure 4C) and TG (Figure 4D) increased by 47% and 13% respectively in the CRS group compared to the intact control. Diazepam, aqueous and methanolic extracts all improved the lipid profile of stressed dams by increasing HDL level up to 51%, 47% and 43% respectively, as compared to the DW control. In addition, the methanolic extract lowered TG level (47%) and the atherogenic index (56%) of stressed dams. Treatment of unstressed pregnant dams with plant extracts improved the lipid profile, with significant reduction of the atherogenic index (31%, $p< 0.05$) compared to the intact control.

Effects of P. muellerianus on placental oxidative and nitric oxide

Figure 5 shows that CRS significantly increased placental MDA (Figure 5A, $p< 0.05$) level and reduced SOD activity by 41% (Figure 5B) in the DW group compared to the intact group. A non-significant increase of GSH (Figure 5C, 62%) was observed in the CRS group in relation to the intact group. Similar to diazepam, plant extracts prevented oxidative stress, as evidenced by reduced MDA ($p< 0.05$-0.01) and increased SOD (AE60: 24%, ME372: 44%) compared with the CRS control. CRS induced a decreasing trend (31%) of placental total proteins (Figure 5D) in the DW group that was improved by all treatments compared to the intact group. In unstressed pregnant rats, plant extracts maintained low the MDA (AE372: $p< 0.05$) and protein levels, and increased SOD activity ($p< 0.001$) compared to the intact group. Placental NO was elevated (Figure 5E, $p< 0.001$) in the CRS group compared to the control group. Diazepam or aqueous extract significantly ($p< 0.001$) lowered NO level in comparison to CRS control. In unstressed pregnant rats, plant extracts maintained low ($p< 0.001$) this parameter as in the intact control.

Effects of P. muellerianus on pro-inflammatory cytokines

Placenta and plasma pro-inflammatory cytokines (IL-1β, TNF-α) levels are presented in Figure 6. A significant elevation of both IL-1β ($p< 0.01$) and TNF-α ($p< 0.001$) was observed in the placenta of the CRS+DW group compared to the intact control (Figure 6A and 6C). In the plasma, a non-significant reduction of both IL-1β (23%) and TNF-α (50%) in the DW group compared to the intact one was noted (Figure 6B and 6D). An important prevention of placenta inflammation was witnessed in diazepam (IL-1β: $p< 0.01$), aqueous (IL-1β: $p< 0.01$) or methanolic (TNF-α: $p< 0.05$) extracts groups compared to the DW control. Conversely, plasma cytokine levels did not change after diazepam or plant extract treatments. In unstressed pregnant dams, plant extracts maintained placenta cytokines like in the intact control.

Discussion

Intrauterine growth restriction (IUGR) remains a major challenge for obstetricians and pediatricians, as it exposes the fetus to low birth weight and heightened postnatal risks [34, 35]. This study investigated the protective effects of *Phyllanthus muellerianus* against chronic restraint stress (CRS)-induced intrauterine growth restriction (IUGR) and to explore the underlying mechanisms involved. Importantly, we report for the first time that *Phyllanthus muellerianus* mitigates CRS-induced IUGR, as evidenced by improvements in fetal growth indices and maternal reproductive outcomes. These beneficial effects may be mediated at least in part, by the enhancement of maternal progesterone levels and the attenuation of placental oxidative stress and inflammation.

To assess the preventive effects of this plant on placental function and fetal development, the restraint stress model was used to induce pregnancy complications [36, 37]. Stress was applied as from gestational days 7 to 18, which correspond to the fetal development period [38]. Available results showed that chronic restraint stress (CRS) in pregnant females resulted in a 30% reduction in body weight, coupled to low ovarian and uterine weights compared to the control group. Plant extracts treatment did not influence body weight but improved ovarian and uterine weights. Weight loss is reported to be associated to reduced food intake, potentially through hypothalamic appetite-regulating hormones such as leptin [39]. It appears that *P. muellerianus* does not stimulate appetite but interfere with reproductive organs growth as previously reported [26]. During CPS, glucocorticoid levels increase following hypothalamic activation of corticotropin-releasing hormone (CRH). In this study, stressed dams showed a 24% rise of corticosterone, which was reduced by the anxiolytic drug DIA, but unaffected by the plant. This aligns with Kaya et al., [11], although corticosterone may vary according to circadian rhythm, stress intensity and duration. Additionally, elevated corticosterone can impair placental barrier function, potentially disturbing the fetal intrauterine environment and maternal reproductive outcomes [12].

In this line, a 22% maternal mortality rate was observed in the stressed dams. CRS impaired maternal reproductive outcomes, as evidenced by a reduction in the number of implantation sites, mean live fetuses, fetal viability and increased post-implantation loss. Plant extracts, especially the methanolic extract enhanced reproductive outcomes and unchanged mortality rate during CRS. These findings suggest that *P. muellerianus* mitigates maternal toxicity, thereby supporting the implanted blastocysts for a proper development. Furthermore, activation of the maternal hypothalamic-pituitary-adrenal (HPA) axis can impair progesterone signaling and reduce uterine blood flow, ultimately contributing to alterations in fetal development [40]. This study showed a significant reduction of the fetal mean weight and fetal crown-rump length (CRL) in stressed dams as compared to the control, confirming IUGR traits. Supplementation with plant extracts reduced the risk of IUGR by increasing fetal mean weight (29%) and CRL (26%). Similar results were reported with natural products such as *Brassica oleracea* or Folic acid using various models of IUGR [6, 8]. The present findings demonstrate that *P. muellerianus* extracts, administered at the dose of 372 mg/kg (corresponding to the recommended human-equivalent dose) can effectively alleviate CRS-induced IUGR.

Different stressors are shown to alter sex hormones regulation, primarily progesterone whose low level can predict miscarriage [41, 42]. Present results show that stress-related suppression of progesterone release is not associated with changes in 17β-estradiol level. Globally, plant extracts significantly boosted progesterone level during CRS or its absence. This important finding suggests that progesterone-like effect of *P. muellerianus* may be sustained by the placenta during organogenesis to maintain pregnancy. Some authors pointed out energy balance to contribute to changes in progesterone [10]. Maternal energy balance marked by plasma lipid profile showed an increase in LDL along with a reduction of HDL, TG and the atherogenic index in stressed dams. This result suggests that lipid accumulation or dyslipidemia can influence fetal metabolic function [43]. Globally, plant extracts improved the lipid profile by reducing TG and increasing HDL. *Phyllanthus* species are known to counteract dyslipidemia due to the presence of gallic acid and genistein [22, 44]. Fetal growth depends heavily on the placenta, a critical temporal organ which interacts with the intrauterine environment [7].

Placental efficiency defined as the ratio of fetal on placenta weights was reduced in IUGR cases from this study, suggesting abnormal placental development [45]. The methanolic extract of *P. muellerianus* reversed the CRS-induced decline in placental efficiency, despite no change on placental weight. Comparable results reported by Zhang et al., [8] showed that fetal abnormal development is not necessarily associated to placental weight loss. The high metabolic activity in the placenta makes it vulnerable to oxidative stress [46, 47]. The rise in the lipid peroxidation product (MDA) and reduction of superoxide dismutase (SOD) observed in stressed subjects indicate placenta cells oxidative damages. Additionally, nitric oxide level was markedly elevated in stressed dams, suggesting inflammation in placental cells. Similar IUGR models have identified oxidative stress in the placenta as the cause of vascular dysfunction in the labyrinth zone [46]. *P. muellerianus* effectively mitigated OS, potentially triggered by antioxidant compounds (nitidine and linoleic acid) previously shown in this plant [48]. Folic acid showed consistent result, as it upregulated antioxidant enzymes via the SIRT/Nrf2 signalling pathway [8]. Moreover, plant extracts could lower NO level, suggesting vasodilation, anti-inflammatory and pro-angiogenesis in the placenta. But the implication of either eNOS or iNOS is needed to confirm this finding. Pro-inflammatory cytokines are regularly mobilized in response to the detrimental effects of OS. In the placenta, the main mediator of inflammation NF-κB stimulates pro-inflammatory cytokines synthesis [49]. CRS significantly increased placental IL-1β and TNF-α without marked changes in the maternal plasma concentrations. This denotes tissue injury and not maternal toxicity as factors responsible for fetal inflammation. Plant extracts efficiently reversed inflammation, suggesting that the anti-inflammatory properties mediate proper fetal growth. Genistein with anti-inflammatory properties were identified in this plant, and therefore justify the present findings [21]. Consistent findings were obtained using *Salvia miltiorrhiza*, which mitigated retarded fetal growth through their antioxidant and anti-inflammatory properties [50].

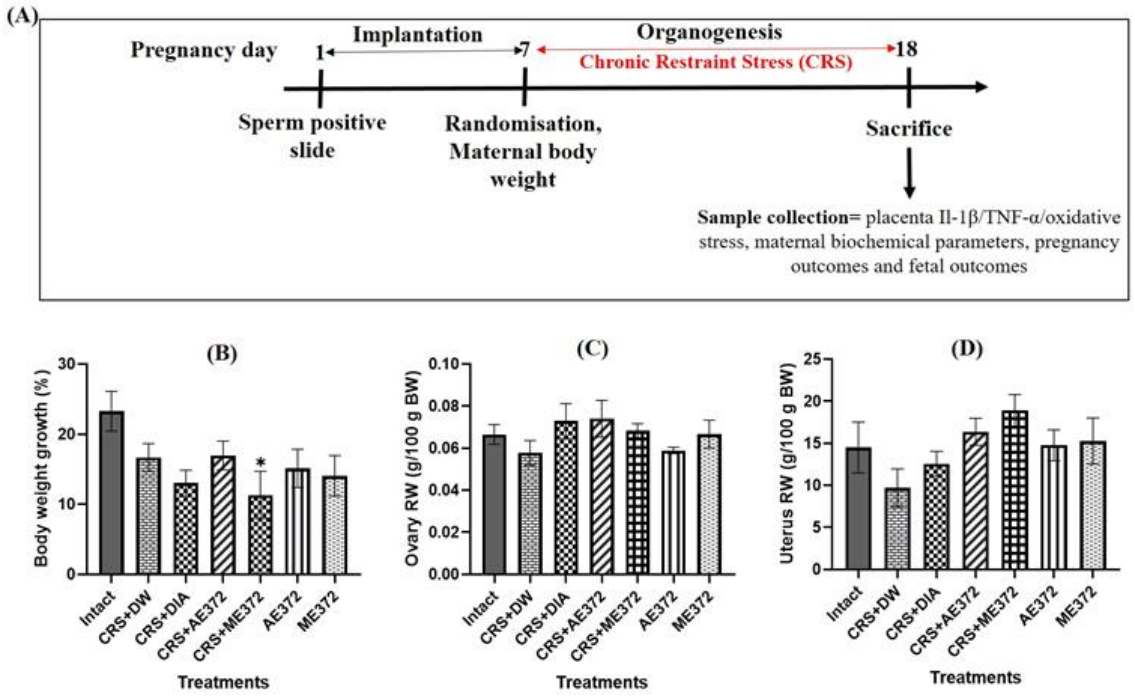

Figure 1. Experimental setup (a), body mass growth (b), ovary and uterus relative weights (c and d) of pregnant rats exposed to CRS.
Values are expressed as mean ± SEM. Number of rats/group = 5; *: p< 0.05 significantly different compared with intact group. DW: distilled water; DIA: Diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

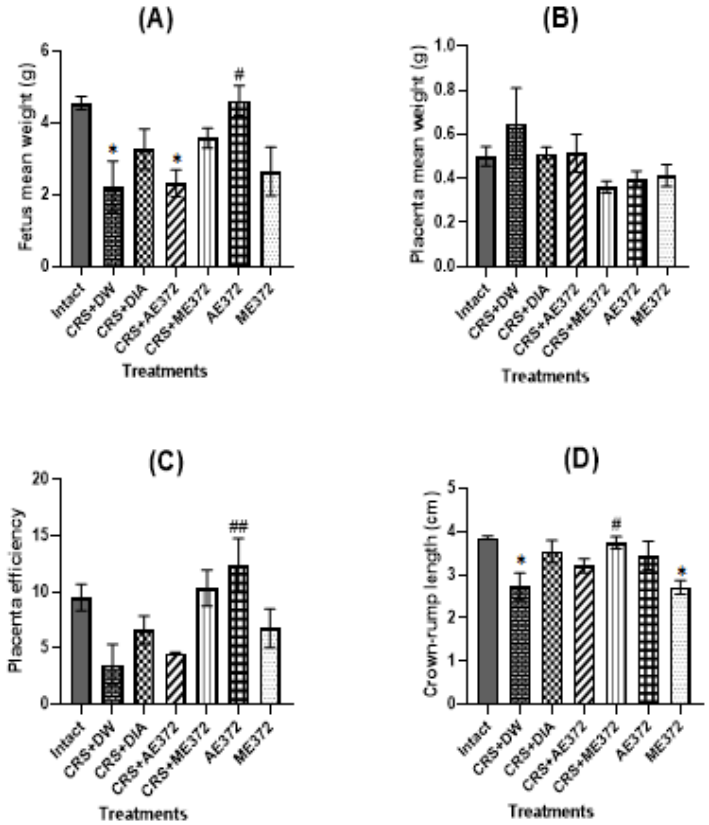

Figure 2. Fetal growth indices after CRS and *P. muellerianus* treatments. (a) Fetal mean weight. (b) Placenta mean weight. (c) Placenta efficiency. (d) Crown-rump length.
Values are expressed as mean ± SEM. Number of rats/group = 5; *: p< 0.05 significance difference with Intact pregnant rats. #: p< 0.05; ## *p* < 0.01 significance difference with CRS+DW. CRS: Chronic restraint stress; DW: distilled water; DIA: Diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

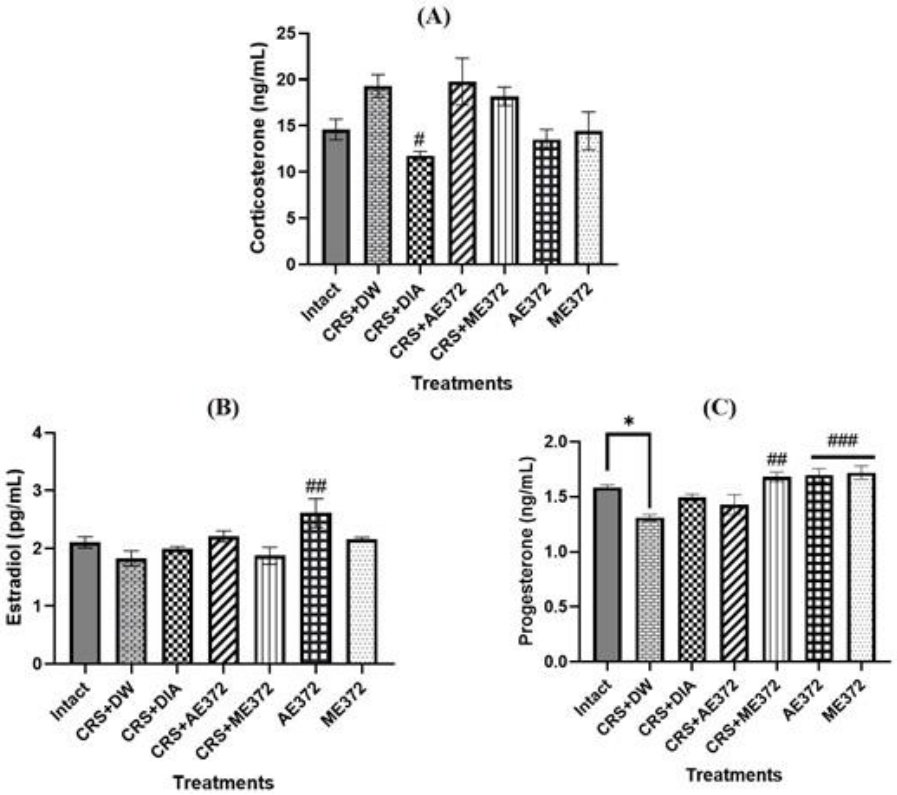

Figure 3. Maternal plasma corticosterone (a), 17β-estradiol (b) and progesterone (c) levels in pregnant dams treated with *P. muellerianus*.
Values are expressed as mean ± SEM. Number of rats/group = 5. *: p< 0.05; significance difference with intact group. #: p< 0.05; ##: p< 0.01; ###: p< 0.001; significantly different compared to CRS+DW. CRS: Chronic Restraint Stress; DW: distilled water; DIA: diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

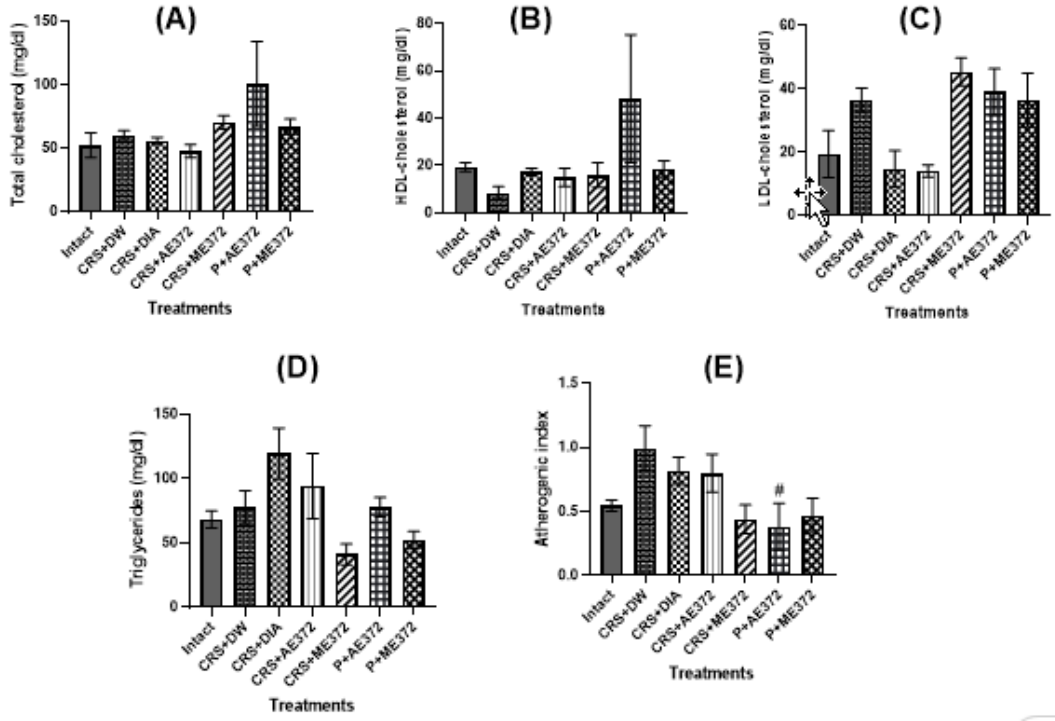

Figure 4. Maternal plasma TC (a), HDL (b), LDL (c), TG (d) and atherogenic index in pregnant dams treated with *P. muellerianus*.
Values are expressed as mean ± SEM. Number of rats/group = 5; #: p< 0.05; significantly different to CRS+DW. CRS: Chronic restraint stress; DW: distilled water; DIA: diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

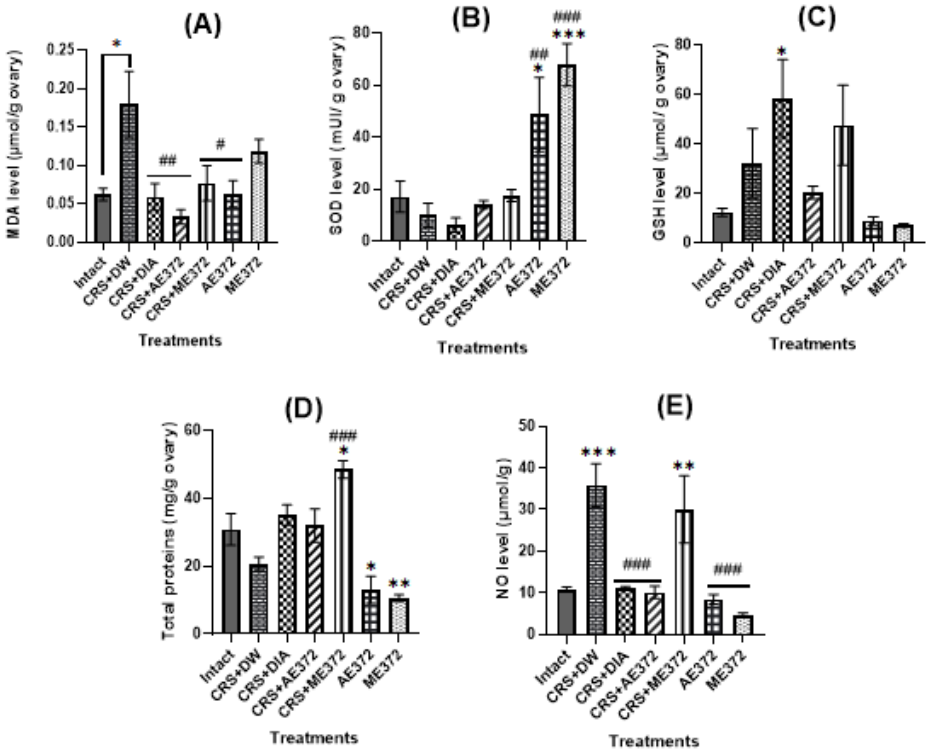

Figure 5. Placenta oxidative stress, total proteins and NO levels in CRS pregnant dams treated with *P. muellerianus*.

(a) Malondialdehyde (MDA). (b) Superoxide dismutase (SOD). (c) Reduced glutathione (GSH). (d) Total proteins. (e) Nitric oxide (NO) level.

Values are expressed as mean ± SEM. Number of rats/group = 5; *: p< 0.05; **: p< 0.01; ***: p< 0.001; significance difference with intact group. #: p< 0.05; ##: p< 0.01; ###: p< 0.001; significance difference with CRS+DW. CRS: Chronic restraint stress; DW: distilled water; DIA: Diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

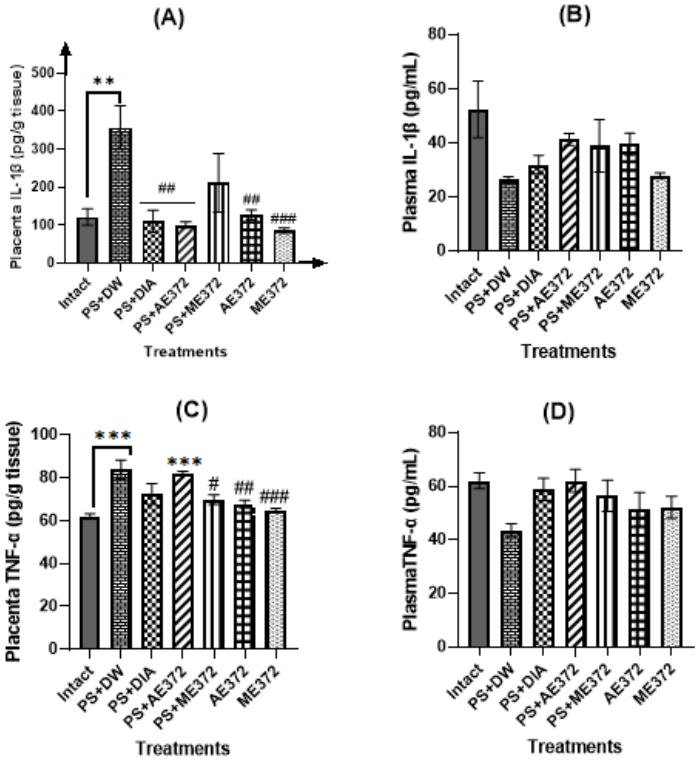

Figure 6. Placenta and plasma pro-inflammatory cytokines level following CRS and *P. muellerianus*.

(A and B): Placenta and plasma IL-1β (C and D): Placenta and plasma TNF-α. Values are expressed as mean ± SEM. Number of rats/group = 5; **: p< 0.01; ***: p< 0.001; significance difference with intact group. #: p< 0.05; ##: p< 0.01; ###: p< 0.001; significance difference with CRS+DW. CRS: Chronic restraint stress; DW: distilled water; DIA: Diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

Table 1. Reproductive outcomes after chronic restraint stress exposure and treatments with *P. muellerianus.*

Treatments	Intact	CRS+DW	CRS+DIA	CRS+AE372	CRS+ME372	AE372	ME372
Mortality rate n (%)	0/7 (0%)	7/9 (22.2%)	5/7 (28.6%)	5/7 (28.6%)	6/7 (14.6%)	0/6 (0%)	0/6 (0%)
Number of Implants	8.00 ± 1.00	6.00 ± 1.18	6.00 ± 0.95	8.4 ± 1.21	8.6 ± 0.93	8 ± 0.45	8 ± 0.63
Number of Resorptions	0	1.2 ± 0.58	0.6 ± 0.4	0.6 ± 0.4	0	0	0
Mean Live Fetuses	8.00 ± 1.00	4.8 ± 0.68	5.4 ± 0.6	7.8 ± 1.5	8.6 ± 0.93	8 ± 0.45	8 ± 0.63
Post-implantation loss (%)	0	16.57 ± 7.95	7.3 ± 4.64	11.82 ± 9.71	0	0	0
Fetal viability (%)	100	83.43 ± 7.95	92.7 ± 4.64	88.18 ± 9.71	100	100	100

Values are expressed as mean ± SEM. Number of rats/group = 5. CRS: Chronic Restraint Stress; DW: distilled water; DIA: diazepam at 3 mg/kg; AE372: aqueous extract at 372 mg/kg; ME372: methanol extract at 372 mg/kg.

Conclusion

This study demonstrates that *P. muellerianus* exerts protective effects against chronic restraint stress-induced IUGR, likely through its antioxidant, anti-inflammatory, progesterone-like activities, as well as its ability to improve maternal reproductive health. These results suggest its potential to alleviate pregnancy complications and reduce the risk of neonatal mortality. The effects of these plant extracts on placenta histology and materno-fetal barrier remain a limitation to this study. Future research is needed to clarify the mechanisms of action of these extracts and evaluate the clinical applicability for the development of new therapies targeting IUGR.

Abbreviations

IUGR: Intrauterine growth restriction
CRS: Chronic restraint stress
MDA: Malondialdehyde
SOD: Superoxide dismutase
GSH: Reduced glutathione
ROS: Reactive oxygen species
NF-κB: Nuclear factor kappa beta
HPA: Hypothalamic-pituitary-adrenal axis
HPO: Hypothalamic-pituitary-ovarian axis
CRH: Corticotropin releasing hormone
eNOS: Endothelial nitric oxide synthase

Authors' Contribution

HHKZN: Conceptualization, Investigation, Methodology, Data analysis and processing, Writing–original draft, Writing–review & editing. AJN: Methodology, Data analysis and processing, Manuscript review & editing. GRBF: Methodology, Writing–review & editing, Validation. MS: Data analysis and processing, Writing–review & editing. PCNN: Investigation, Methodology, manuscript draft. ACMT: Writing-original draft, Writing–review & editing. PW: Conceptualization, supervision, Writing–original draft, Writing–review & editing. All authors reviewed and approved the final version of the manuscript

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Conflict of interest

The authors declare no conflict of interest

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