

The Impact of administration of *Annona muricata* leaf on estrogen and progesterone functions following mercury-induced toxicity in adult female Wistar rats

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World Journal of Advanced Research and Reviews, 2024, 21(02), 1357–1363

Publication history: Received on 08 January 2024; revised on 17 February 2024; accepted on 20 February 2024

Article DOI: <https://doi.org/10.30574/wjarr.2024.21.2.0545>

Abstract

Environmental toxicants have been linked to female reproductive dysfunction resulting from oxidative stress. The study assesses the impact of ethanolic leaf extract of *Annona muricata* (EAM) on ovarian and uterine function following mercury-induced toxicity in female Wistar rats. The study involved 40 female Wistar rats, each weighing 114–156g, divided into eight groups of five animals each. Group A received feed and water only; group B received 500 mg/kg of EAM only; and group C received 25 mg/kg of mercury chloride (HgCl₂). Groups D and E received 100 mg/kg and 500 mg/kg of EAM for 4 weeks and were treated with 25 mg/kg of HgCl₂, respectively. Group F received 500 mg/kg of EAM for 4 weeks and treated immediately with co-administration of 25 mg/kg of HgCl₂ and 500 mg/kg of EAM for 4 weeks. Groups G and H received 25 mg/kg of HgCl₂ and received 100 mg/kg and 500 mg/kg of EAM for 4 weeks, respectively. Progesterone and Estrogen levels were measured at the end of the administration. The data was analysed using SPSS version 25, with inferential statistics performed, and significant values were set at p≤0.05. Administration of mercury resulted in a decreased level of estrogen, and progesterone. EAM treatments resulted in statistically significant increased levels of progesterone in groups E, F, G and H; group D also showed an increase, though not statistically significant. Estrogen levels were increased in groups F, G and H, but groups D and E showed a decrease in estrogen levels, though not statistically significant. The study reveals that EAM enhances female reproductive hormonal levels and may turn out into a potential capable of reversing or preventing female infertility linked to mercury chloride toxicity.

Keywords: Estrogen; Progesterone; *Annona muricata*; Mercury Chloride; EAM

1. Introduction

The reproductive system in females is responsible for the production of gametes (called eggs or ova), certain sex hormones, and the maintenance of fertilized eggs as they develop into a mature fetus and become ready for delivery (Rosner and Sarao, 2019). The ovaries are female gonads in which gametogenesis occurs and are responsible for the secretion of sex hormones. Progesterone is a steroid hormone that is responsible for preparing the endometrium for uterine implantation of the fertilized egg. If a fertilized egg implants, the corpus luteum secretes progesterone in early pregnancy until the placenta develops and takes over progesterone production for the remainder of the pregnancy (Holesh *et al.*, 2021; Holesh and Lord, 2018). Progesterone and estrogen are key regulators of myometrial growth, contractility, and uterine peristalsis. Progesterone and estrogen play an important role in determining the contractile state of the pregnancy myometrium and the timing and process of parturition (Sajadi *et al.*, 2018). Vaisbuch, Erez, and Romero (2015) reported that progesterone works in synergy with estrogen in the development and sexual maturation of the reproductive organs and coordinates the menstrual cycle. It is a key hormone that plays a role in the preparation of pregnancy (the uterine decidua, myometrium, and cervix during the menstrual cycle through blastocyst

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implantation) and maintenance, sustaining myometrium quiescence, cervical competence, and modulation of the maternal immune system during pregnancy (Cable and Grider, 2022).

Mercury is present in various forms (organic, inorganic, and elemental) and is primarily absorbed by humans through seafood and sashimi consumption. It can also be found in dental amalgams, broken thermometers, fluorescent light bulbs, button-cell batteries, and skin-lightening creams (Capcarova *et al.*, 2019). Mercury exposure is linked to its environmental concentrations, which can vary due to industrial activities and natural sources like volcanic eruptions and rock weathering. Additionally, mercury can undergo biomagnification, accumulating in the food chain and causing higher concentrations in predatory fish and seafood consumed by humans (Massányi *et al.*, 2020). Reports have shown that mercury intake has resulted in the inhibitory release of LH and FSH from the anterior pituitary gland, which resulted in hormonal imbalance leading to polycystic ovarian syndrome (Henriques *et al.*, 2019). A study demonstrated that exposure to mercury led to increased levels of estrogen and progesterone, causing painful or irregular menstruation, tipped uterus, premature menopause, and often different ovarian dysfunctions (Chen *et al.*, 2006; Massányi *et al.*, 2020). Mercury exposure has been shown to decrease progesterone levels, reduced glutathione, catalase, and superoxide dismutase activity, with FSH and LH having an inverse correlation with mercury doses (Ma *et al.*, 2018). Altunkaynak *et al.* (2016) reported an alteration in the ovarian histology revealing hemorrhage, edema, and reduced ovarian follicles following mercury exposure.

Annona muricata L. (Magnoliales: Annonaceae) is a tropical plant species known for its edible fruit, which has some medicinal merits but also some toxicological effects. *A. muricata* has been shown to possess anxiolytic, anti-stress, anti-inflammatory, contraceptive, anti-tumoral, anti-ulceric, wound healing, hepatoprotective, anti-icteric, and hypoglycemic activities (Coria-Téllez *et al.*, 2018). It contains bioactive chemical properties such as alkaloids, acetogenins, flavonoids, terpenes, and essential oils (Zubaidi *et al.*, 2023); these properties contribute to its potential therapeutic uses in various diseases and disorders. Olowofolahan *et al.*, (2022) reported an improved levels of estrogen, LH, and FSH, following ingestion of *Annona muricata* stem bark in estradiol benzoate toxicity. Despite, reports on mercury toxicity on ovarian functions demonstrating different ovarian etiologies such as polycystic ovarian function, hormonal imbalance; there are limited pieces of literatures on the impact of ethanolic leaf extract of *Annona muricata* on mercury toxicity in progesterone and estrogen function, which this study investigates. The study was aimed at determining the impact of administration of *Annona muricata* leaf on progesterone and estrogen function following mercury-induced toxicity in adult female Wistar rats.

2. Material and methods

2.1. Area of Study

The study was carried out in the Department of Human Anatomy, Animal House, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, College of Health Sciences, Nnewi Campus.

2.2. Ethical Approval

Ethical approval was obtained from the Faculty of Basic Medical Science, College of Health Science, Nnamdi Azikiwe University, Nnewi campus. Rats handling and treatments conform to the National Institute of Health guidelines for laboratory animal care and use (Carbone and Austin, 2016). The ethical approval number is NAU/CHS/NC/FMBS/691.

2.3. Procurement and Identification of *Annona muricata*

Leaves of *Annona muricata* (Soursop) leaves was harvested from a local farm in Okofia Community, Nnewi, Otolo Anambra State. The Department of Botany, Nnamdi Azikiwe University, Awka, Anambra State, identified it and deposited it with a herbarium number NAUH-04B.

2.4. Extract Procedure of *Annona muricata*

Annona muricata (Soursop) leaves were washed in running tap water to remove dirt, and shed-dried under ambient temperature. The dried leaves of *Annona muricata* (Soursop) were milled into a coarsely powdered form using a local grinder. 250g of the dried leaf was macerated in 1000mls of 95% absolute ethanol for 48hours. It was filtered using a clean handkerchief and further filtration using Whatman No 1 filter paper. The filtrate was concentrated using a rotatory evaporator and dried further using a laboratory oven at 45 degrees centigrade into a gel-like form. The extract was preserved in airtight container and kept in a refrigerator for further usage. The extraction method was done with modifications as described according to the method employed by Al-Attar and Abu Zeid (2013).

2.5. Acute Toxicity of *Annona muricata* and Mercury Chloride

The median lethal dose (LD50) of the Ethanolic leaf extract of *Annona muricata* and mercury chloride was determined using Lorkes method (1983), and it was divided into two-phase. This was conducted in the Department of Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus. In this study, 13 rats were used for the Ethanolic leaf extract of *Annona muricata* and mercury chloride were administered via oral route and carried out in two phases. The median lethal dose for leaf extract of *Annona muricata* was found to be above 5000mg/kg, and that of mercury chloride; 75mg/kg.

2.6. Experimental Animals

Forty-(40) female Wistar rats weighing 114-156 gram were obtained from the Animal House, Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus. Animals were kept in standard cages at a room temperature of $27\pm 2^{\circ}\text{C}$. The animals were maintained with normal laboratory chow and water *ad libitum*. The animals were acclimatized for two weeks, before administering the ethanolic leaf extract of *Annona muricata* (EAM) and Mercury chloride.

2.7. Experimental Design

The animals were divided into eight groups of five animals each as indicated below:

- Group A received feed and water only *ad libitum* and served as positive control
- Group B received 500 mg/kg of EAM only
- Groups C received 25 mg/kg of mercury only
- Group D received 100 mg/kg of EAM for 4-weeks and treated immediately with 25 mg/kg of mercury chloride for 4-weeks
- Group E received 500 mg/kg of EAM for 4-weeks and treated immediately with 25 mg/kg of mercury chloride for 4-weeks
- Group F received 500 mg/kg of EAM for 4-weeks and treated immediately with co-administration of 25 mg/kg of mercury and 500 mg/kg of EAM for 4-weeks
- Group G received 25 mg/kg of mercury for 4-weeks and treated immediately with 100 mg/kg of EAM for 4-weeks
- Group H received 25 mg/kg of mercury for 4-weeks and treated immediately with 500 mg/kg of EAM for 4-weeks

All experimental protocols were observed under strict supervision, the experiment lasted for eight-weeks, and administration was done through oral gavage.

2.8. Sample Collection

Twenty-four hours after the administration, animals in the different groups were anesthetized using chloroform in an enclosed container. Blood samples were collected from the animals using a heparinized capillary tube through ocular puncture as described by Parasuraman, Raveendran, and Kesavan (2010). Blood obtained was put in a plain bottle, allowed to clot, and centrifuged for 10-minutes at 3000rpm, after which the serum was retrieved using a micropipette. The retrieved serum was used to assay for hormonal profile (progesterone and estrogen,).

2.9. Progesterone and Estrogen Level

Serum samples retrieved were used to assay progesterone and estrogen using the enzyme immunoassay (EIA) technique as described by the Manufacturer's manual.

2.10. Principle of Progesterone

The samples and progesterone enzyme conjugate are added to the wells coated with anti-Progesterone monoclonal antibody. Progesterone in the sample competes with a progesterone enzyme conjugate for binding sites. Unbound progesterone and progesterone enzyme conjugate are washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of progesterone in the samples. A standard curve is prepared relating color intensity to the concentration of the progesterone. It will be recorded in ng/mL.

2.11. Procedure

The desired number of coated strips were placed into the holder and 10 mL of Progesterone standards, control, and serum samples was pipetted. Thereafter, 200 mL of Progesterone Enzyme Conjugate was added to all wells and incubated for 60 minutes at room temperature (18–26 °C). Liquid was removed from all wells. Wells were washed three times with 300 mL of 1x wash buffer. Blot on absorbent paper towels, and 100 mL of TMB substrate was added to all wells, and it was incubated for 15 minutes at room temperature. 50 mL of Stop Solution was added to all wells. The plate was shaken gently to mix the solution, and the absorbance on ELISA Reader was read at 450 nm within 15 minutes after the Stop Solution was added.

2.12. Principle of Estrogen

The samples and estrogen enzyme conjugate were added to the wells coated with anti-estrogen monoclonal antibody. Estrogen in the sample competes with a progesterone enzyme conjugate for binding sites. Unbound estrogen and estrogen enzyme conjugate are washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of estrogen in the samples. A standard curve is prepared relating color intensity to the concentration of the estrogen. It will be recorded in pg/mL.

2.13. Procedure

The desired number of coated strips were placed into the holder and 10 mL of estrogen standards, control, and serum samples was pipetted. Thereafter, 200 mL of estrogen Enzyme Conjugate was added to all wells and incubated for 60 minutes at room temperature (18–26 °C). liquid was removed from all wells. Wells were washed three times with 300 mL of 1x wash buffer. Blot on absorbent paper towels, and 100 mL of TMB substrate was added to all wells, and it was incubated for 15 minutes at room temperature. 50 mL of Stop Solution was added to all wells. The plate was shaken gently to mix the solution, and the absorbance on ELISA Reader was read at 450 nm within 15 minutes after the Stop Solution was added.

2.14. Statistical Analysis

Data obtained from this study was analyzed using Statistical Package for Social Sciences (SPSS) version 25. Data was considered significant at $p \leq 0.05$.

3. Results

Table 1 Effect of ethanolic leaf extract of *Annona muricata* on E2 and PG2 following mercury chloride toxicity

	Estrogen level	Progesterone level (ng/ml)
	Mean±SEM	Mean±SEM
Group A (control)	50.0±1.15*	47.66±1.45*
Group B (500 mg/kg of EAM)	26.66±3.33	15.66±2.33*
Group C (25mg/kg of HgCl ₂)	30.00±2.88 ^a	30.0±2.88 ^a
Group D (100mg/kg of EAM + 25mg/kg of HgCl ₂)	27.66±6.74	33.33±1.66
Group E (500mg/kg of EAM + 25mg/kg of HgCl ₂)	27.33±1.45	37.33±1.45*
Group F (500mg/kg of EAM + co-administered 25mg/kg of HgCl ₂ and 500mg/kg of EAM)	44.00±1.00*	39.66±0.88*
Group G (25mg/kg of HgCl ₂ + 100 mg/kg of EAM)	48.00±2.00*	41.00±2.08*
Group H (25mg/kg of HgCl ₂ + 500 mg/kg of EAM)	63.33±1.66*	44.33±2.33*
F-ratio	19.72	25.61

Data was analyzed using ANOVA followed by post LSD multiple comparison and values were considered significant at $p < 0.05$. SEM: standard error of mean, HgCl₂: mercury chloride, EAM: ethanolic leaf extract of *Annona muricata*, ^a: significant when compared with group A (control), *: significant when compared with group C.

The mean Estrogen indicated that group C ($p=0.00$) had a low statistically significant difference when compared with the control group (A). The data also showed that groups F, G and H ($p=0.00$, $p=0.00$, $p=0.00$, $p=0.00$) had a high statistically significant difference when compared to group C while groups B, D and E had no statistically significant difference when compared to group C. The mean Progesterone indicated that group C ($p=0.00$) had a low level statistically significant difference when compared with the control group (A). The data also showed that groups E, F, G and H ($p=0.03$, $p=0.01$, $p=0.00$, $p=0.00$, $p=0.00$) had a high statistically significant difference and group B ($p=0.00$) had a low statistically significant difference when compared to group C while group D had no statistically significant difference when compared to group C.

4. Discussion

The study result revealed a significant decline in the estrogen level following the exposure of experimental rats to mercury chloride as indicated in group C compared to control. However, the physiology linked to the decrease in the estrogen level is probably as a result of increased production of free radical species such as ROS and reactive nitrogen species, which inhibits the release and synthesis of estrogen. The reports of Davis *et al.*, (2001), Chen *et al.* (2006), Massányi *et al.* (2020) demonstrated a significant increase in the estrogen level following mercury intoxication in experimental animals, which is in contrast to these study findings. Further, the study aligns with the works of Zhang *et al.*, (2016), Crump and Trudeau, (2009) revealing a significant reduction in the estrogen level following mercury toxicity. However, treatments with EAM at the pre and post mercury exposure showed significant increase in the estrogen level in the post treatment with EAM as indicated in groups G and H while group F a co-administrative group indicated significant increase as well. Further, the physiological changes associated with the significant increase is probably linked to flavonoids present, which has the potency of combating ROS formation, which inhibits the synthesis and release of estrogen hormones. Also, the EAM pre-treatment showed a non-significant decrease in the estrogen level post treatment with mercury compared to mercury chloride alone, the physiology linked to these changes could result from increased lipid peroxidation from mercury metabolism, which tends to alter the rate, synthesis and release of estrogen.

The study revealed that mercury exposure produced a significant decrease in the progesterone level as shown in group C compared to control group. The reason for the significant decline in progesterone level is probably as a result of increased level of ROS formation following the mercury toxicity, which alters the progesterone synthesis. The study agrees with the findings of Ma *et al.*, (2018), Zhu, Kusaka, Sato, and Zhang (2000), Bjørklund *et al.*, (2019) indicating a significant decline in the progesterone level following mercury toxicity. Davis *et al.*, (2001), Koli, Prakash, Choudhury, Mandil, and Garg, (2020) reported a non-significant decrease in the progesterone level following mercury toxicity in female rats. However, treatment with EAM, pre and post mercury treatment and co-administrative treatment following mercury exposure resulted in an increase level of progesterone compared to mercury treated group. Further, significance was indicated in groups E, F, G, and H while group D had no significance; thus, the mechanism of action following the significant increase may result from flavonoids impact in the combating ROS formation through complex pathways to regulate the synthesis and release of progesterone to regulate the female ovarian cycles.

Abbreviations

EAM - ethanolic leaf extract of *Annona muricata*

5. Conclusion

The study showed that administration of mercury chloride resulted in decreased estrogen level, and progesterone levels. However, treatments with EAM in the prevent, co-administrative, and curative phase had an improved levels of estrogen, and progesterone levels, which showed significance in the estrogen and progesterone levels

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The ethical committee of Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus, gave approval for the commencement after the authors fully met the requirement of the study, and was monitored to make sure that

all protocols were followed sequentially. An approval number for the commencement of the work was documented by the Head of the ethical committee for reference purposes as stated above in 2.2.

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