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Original Research Article

Uterotrophic bioassay of *Carica papaya* and *Garcinia kola* (bi-herbal) aqueous extract

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Abstract

Purpose: *To investigate the estrogenic effect of Carica papaya, Garcinia kola, bi-herbal (BH) using the uterotrophic assay.*
Methods: *Twenty-five (25) immature female Wister rats (18 days old) were assigned to 5 groups (A – E). Groups A (control group) and E were treated with 10 mL/kg of distilled water and 10 mg/kg estradiol, respectively, while groups B, C and D were given 10, 100 and 1000 mg/kg BH, respectively by oral gavage for 3 consecutive days. The body weight of the animals and the quantity of feed consumed were determined daily. On the 4th day, the rats were anesthetized and blood was collected for estradiol assay using enzyme-linked immunosorbent assay (ELISA) method. The uteri, ovaries and cervix were dissected and weighed. The organs were fixed in Bouins fluid and processed histologically.*
Results: *The result showed an increase in body weight which corresponded with the quantity of feed consumed. There was a non-significant ($p > 0.05$), dose-dependent increase in blood estradiol levels in all BH extract-treated groups and a significant ($p < 0.05$) increase in the estradiol group. Photomicrographs of the uterus, cervix and ovary of all treated groups revealed normal cellular and structural appearance.*
Conclusion: *The aqueous bi-herbal root extract is non-estrogenic at experimental doses. Therefore, it alleviates concerns about infertility and estrogen-induced cancers associated with high estrogen levels with the use of this bi-herbal extract.*

Keywords: *Carica papaya, Garcinia kola, Wister rats, Bi-herbal extract, Uterotrophic assay, Estradiol*

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INTRODUCTION

The uterotrophic assay is an important *in vivo* bioassay for assessing estrogenic or anti-estrogenic substances [1]. Estrogen, a crucial female sex hormone, plays a vital role in regulating uterine tissue growth. Of the 3 types of estrogen, 17β-estradiol (E2) is the primary regulator influencing various aspects of uterine tissue growth [2]. Phytestrogens mimic estrogen by binding to estrogen receptors and regulating genomic expressions. They either activate alpha-estrogen receptors as agonists or inhibit beta-estrogen receptors as antagonists [1]. Central African medicinal plants such as *Eryhtrina lysistemon*, *Brenania brieyi*, *Milletia concaui*, *Eryhtrina poeppigiana*, *Aloe buttneri*, *Justicia insuleris*, *Hibicus macranthus* and *Dicliptera*

verticillata have been reported to possess estrogenic properties [3]. Also, a mild estrogenic effect has been observed in the leaves of *Justicia flava* in immature mice [4]. In addition, the estrogenic-antiestrogenic activities of *Erythrina excelsa* in adult ovariectomized rats have been reported [5].

Garcinia kola, also known as bitter kola, and *Carica papaya*, commonly called pawpaw, are two plants with significant effects on the reproductive system [6,7]. The seed extract of *Garcinia kola* influenced the reproductive cycle and pregnancy in female rats [7]. *Carica papaya* seed and root extracts have been reported to inhibit ovulation in rabbits and cause morphological changes in the uterine endometrial surface of Albino rats [6,8]. Traditionally, combination of *Carica papaya* and *Garcinia kola* roots, has been used in Nigeria to manage heavy or prolonged uterine bleeding and spotting in women of reproductive age.

Given the challenges associated with conventional drug treatments and surgical interventions for uterine disorders, it is essential to explore alternative therapies with improved outcomes. Understanding the estrogenic properties of this combination could provide valuable insights for managing uterine bleeding effectively.

METHODS

Plant material and authentication

Garcinia kola and *Carica papaya* roots were harvested from two communities in Ovia North East Local Government Area, Edo State, Nigeria in November 2020. They were identified and authenticated By Dr. Joseph Erabor of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, with authentication numbers UBH-365 and UBH-C505 for *Garcinia kola* and *Carica papaya,* respectively.

Plant extraction

The roots of the two plants were rinsed with tap water, cut into small pieces and dried under a shade for two weeks. The dried roots were further dried in a hot air oven at 60 ^{o}C for 6 h before pulverizing separately into powder using a laboratory milling machine. Fifty (50) grams each of powdered *Garcinia kola* and *Carica papaya* roots were combined (100 g) and macerated in boiled water. It was left at room temperature (30 ± 2 ^{o}C) with frequent shaking for 72 h followed by filtration using a glass funnel tightly plugged with cotton wool. The filtrate was concentrated in a hot air oven at 60 ^{o}C, the yield was calculated and the concentrate coded “BH” extract. It was properly labeled and kept in the refrigerator at 4 ^{o}C for subsequent use. The yield of the concentrate (Y) was calculated using Eq 1.

$$Y\ (\%) = (W_1/W_2)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

W_1 is the weight of dried extract, and W_2 is the weight of dried powdered root.

Animals

Immature female Wister rats (18 days) were used. The animals were maintained at the Animal Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. Ethical approval (no. EC/FP/021/20) was obtained from the Ethics Committee on the Use of Animals for Experimental Procedures, Faculty of Pharmacy, University of Benin. The animals were housed in well-ventilated polyethylene cages under standard temperature conditions (28 ± 2 ^{o}C), controlled humidity and 12 h-light/12 h-dark cycles. The animals were fed with commercial standard pelleted feed, with free access to clean tap water *ad libitum.* The experiments were performed according to the test guidelines of OCED, 440 (1) and the animals were handled according to the international standards for the care and use of laboratory animals [9]. All animals were weighed on the S-Mettler electronic compact balance model K- 500BH (d = 0.01 g) and weights were recorded in grams. The anal temperature was taken using C-Tone digital thermometer (Mode: GF502) in degrees Celsius.

Uterotrophic assay

Twenty-five (25) healthy female Wister rats (18 days old) were used for this assay. The rats were randomly assigned to 5 groups (A – E; n = 5). Groups A (control group) and E were treated with 10 mL/kg of distilled water and estradiol, respectively and groups B, C and D were given 10, 100 and 1000 mg/kg of BH, respectively. All administrations were given orally by gavage for 3 consecutive days. The animals were weighed and vaginal opening was observed daily at 9.00 am and the quantity of feed consumed daily was also weighed. On day 4, animals were anesthetized with cotton wool soaked in chloroform in a closed system. They were dissected longitudinally over the abdomen. Blood was collected from the abdominal aorta and by cardiac puncture into plain blood sample bottles for estradiol assay. The uterus with the ovaries and cervix was located and excise, adhering

tissues and fat were trimmed off. It was examined macroscopically and weighed (wet weight). The ovaries and cervix were excised from the uterus before fixing in Bouins fluid for histological processing. The tissues (uterus, ovaries and cervix) were dehydrated in ascending grades of ethanol before embedding in paraffin wax, they were sectioned and stained with hematoxylin and eosin. The stained slides were viewed using the Olympus cameral microscope and photomicrographs were taken with x40 objective [1,4].

Determination of estradiol concentration

Enzyme-linked immunosorbent assay (ELISA) is a competitive immunochemistry quantitative assay used for serum blood estradiol levels. Estradiol ELISA kit (Sigma-Aldrich, USA) was used to determine serum estradiol level. Twenty-five microliters of standards, specimens, and controls were dispensed into appropriate wells. The microwells are coated with polyclonal anti-estradiol antibodies. The estradiol in the serum sample competes with the estradiol-horseradish peroxidase conjugate to bind to the coated antibody. The unbound estradiol-horseradish peroxidase conjugate was removed by washing. A solution of tetramethylbenzidine (TMB) reagent was added resulting in the development of a blue colour. The developed colour was stopped with the addition of a stop solution, and the absorbance was measured spectrophotometrically at 450 nm. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The amount of bound estradiol-horseradish peroxidase conjugate is inversely proportional to the concentration of estradiol in the serum sample.

Statistical analysis

Results are presented as a percentage of control in mean ± standard error of the mean (SEM). One-way repeated measures ANOVA with Dunnett's correction for multiple comparisons, or student's *t*-test where appropriate, was used for comparisons. Statistical significance was indicated at $p < 0.05$. GraphPad Prism 9.00 (California, USA) and Microsoft Office Excel were utilized for the analyses.

RESULTS

Uterotrophic assay

There was a daily increase in the quantity of feed (g) consumed by the immature 18-day-old rats in all groups, as shown in Table 1. At the end of the 3 days of oral extract administration, a significant increase ($p < 0.05$) in the body weight of the rats in control group and the group administered 10 mg/kg bi-herbal (BH) extract before and after treatment was observed. However, there was no significant increase in body weight of immature rats in BH extract 100 mg/kg, 1000 mg/kg and estradiol groups before and after treatment (Figure 1). The estradiol group had a significant ($p < 0.05$) increase in relative uterine weight. However, there was no significant difference ($p > 0.05$) in relative uterine weight in BH extract groups (10 mg/kg, 100 mg/kg, and 1,000 mg/kg) compared with control group (Figure 2). On the other hand, the BH extract at 10 mg/kg, 100 mg/kg, and 1000 mg/kg doses did not induce a statistically significant ($p > 0.05$) increase in estradiol blood level but the group given estradiol at 10 mg/kg significantly ($p < 0.05$) increased estradiol blood levels compared with control as shown in Figure 3.

Photomicrograph of the uterus

Photomicrographs of immature rats' uterus revealed normal endometrial glands and endometrial lining supported by normal endometrial stroma (Figure 4 A). The groups treated with BH extract revealed small endometrial glands, and normal endometrial lining supported by a plump endometrial stroma (Figure 4 B, C and D) Furthermore, the group administered estradiol showed normal endometrial glands supported by a plump endometrial stroma (Figure 4 E).

Table 1: Effect of aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* (BH) on average daily feed (g) consumption of immature female rats

Group (dose)	D1	D2	D3	D4	Total	Mean ± SEM
BH (10 mg/kg)	14.0	20.0	20.0	21.0	75.0	18.8±1.6
BH (100 mg/kg)	09.0	13.6	17.2	20.0	59.8	14.9±2.4
BH (1000 mg/kg)	10.9	14.3	18.6	21.3	65.1	16.3±2.3
Estradiol (10 mg/kg)	12.5	15.0	20.0	22.0	69.5	17.4±2.2
Control (distilled water)	17.5	17.7	30.0	31.2	96.4	24.1±3.8

BH = Bi-herbal, D1 = day 1 of treatment, D2 = day 2 of treatment, D3 = day 3 (last day) of treatment and D4 = day of anesthesia. Values are in grams per day

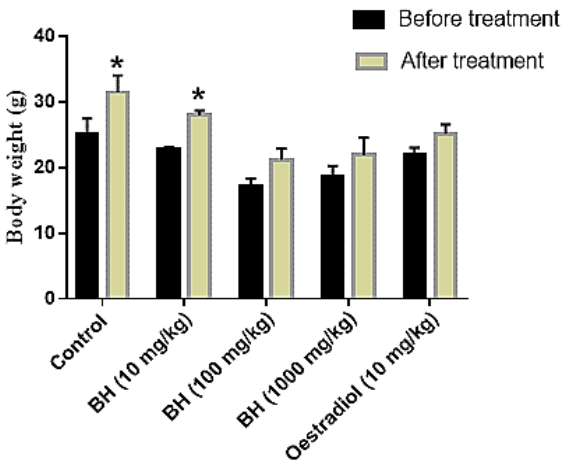

Figure 1: Effect of aqueous bi-herbal (BH) root extract of *Garcinia kola* and *Carica papaya* on body weight of rats. **P* < 0.05 vs control

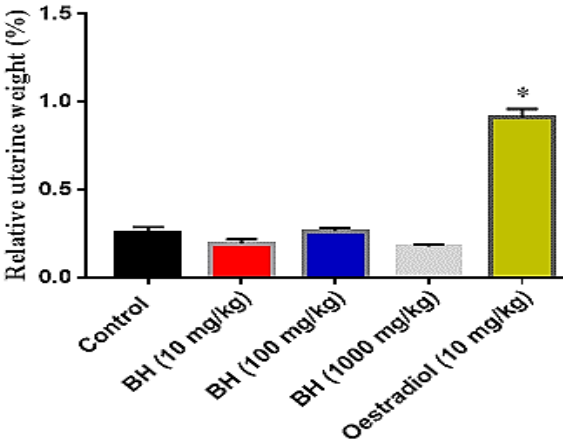

Figure 2: Effect of aqueous bi-herbal root extract of *Garcinia kola* and *Carica papaya* on relative uterine wet weight. ***P* < 0.05 vs control group of immature female rats

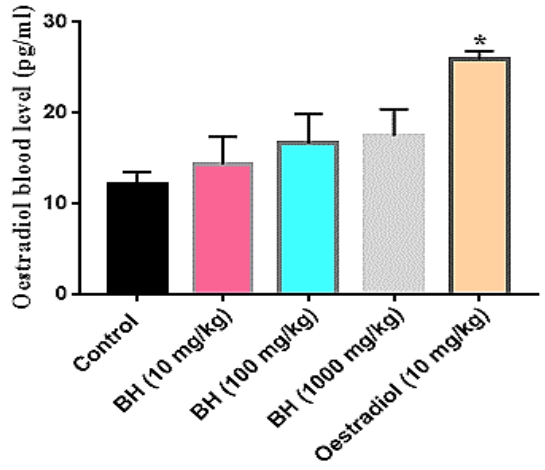

Figure 3: Estradiol blood levels of female immature rats treated with oral administration of BH extract at 10, 100, and 1,000 mg/kg. *P* < 0.05 vs control group

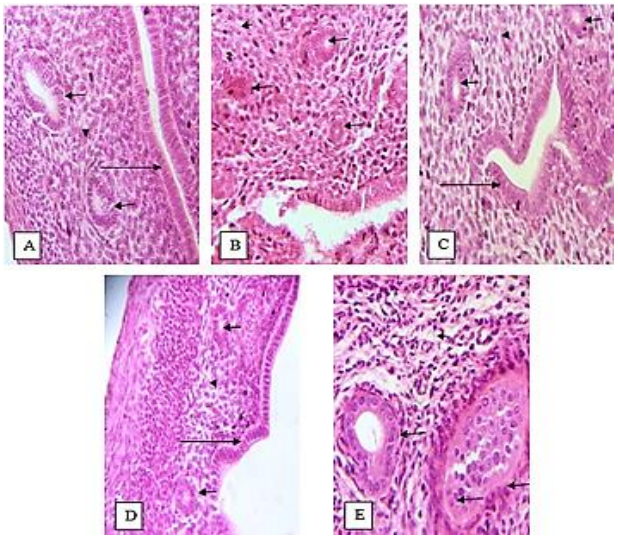

Figure 4: The photomicrographs of the immature female rat uterus stained with H & E (x40).
Key: **A-** Control group showing endometrial glands (short arrow), endometrial lining (long arrow) and endometrial stroma (arrowhead); **B –** BH extract 10 mg/kg, **C – BH** extract 100 mg/kg and **D –** BH extract 1000 mg/kg groups showing small endometrial glands (short arrow) normal endometrial lining (long arrow) and plump endometrial stroma (arrowhead); and **E -** Estradiol group, showing normal endometrial gland (short arrow) and normal stroma (arrowhead)

Photomicrograph of the cervix

Photomicrographs of immature rats' cervix showed normal cervical epithelial lining depicting the transition zone and normal stroma in control group (Figure 5 A). Thickening of the ectocervix and normal stroma was seen in rats treated with BH extract 10 mg/kg (Figure 5 B). However, treatment wth 100 mg/kg of BH extract revealed a normal transition zone of the cervical epithelial lining and plump cervical stroma (Figure 5 C) while a normal endocervical lining and plump endocervical stroma (Figure 5 D) were evident in rats administered BH extract 1000 mg/kg. The ectocervical lining and stroma were normal in rats that were treated with estradiol 10 mg/kg (Figure 5 E).

Photomicrograph of the ovary

Photomicrographs of immature rats' ovaries revealed follicles in normal sequential maturation supported by normal ovarian stroma in control (Figure 6 A). Groups administered BH extract at 10, 100 and 1000 mg/kg body weight (Figure 6 B – D) and the estradiol group (Figure 6 E) revealed follicles in normal sequential maturation surrounded by normal ovarian stroma.

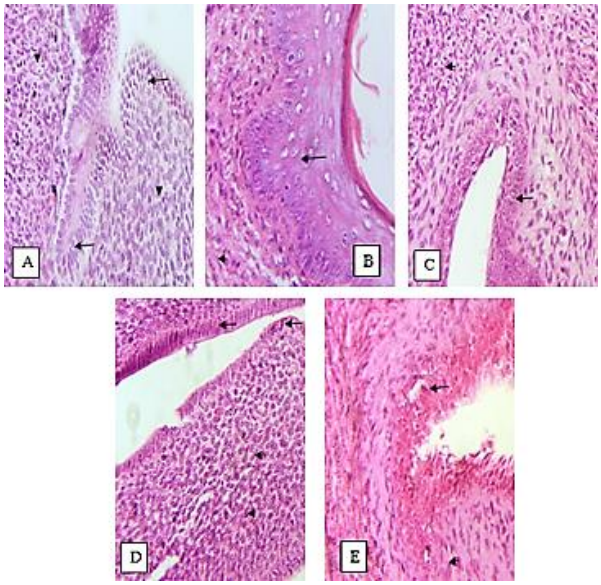

Figure 5: The photomicrographs of the immature female rat cervix stained with H & E (X40). ***Key:*** **A** – Control cervical epithelial lining (short arrow) and stroma (arrowhead); **B –** BH extract 10 mg/kg with thickened ectocervix (short arrow) and stroma (arrowhead); **C** – BH extract 100 mg/kg showing cervical epithelial lining (short arrow) and stroma (arrowhead); **D** – BH extract 1000 mg/kg with endocervical lining (short arrow) and endocervical stroma (arrowhead). **E –** Estradiol 10 mg/kg showing ectocervical lining (short arrow) and stroma (arrowhead)

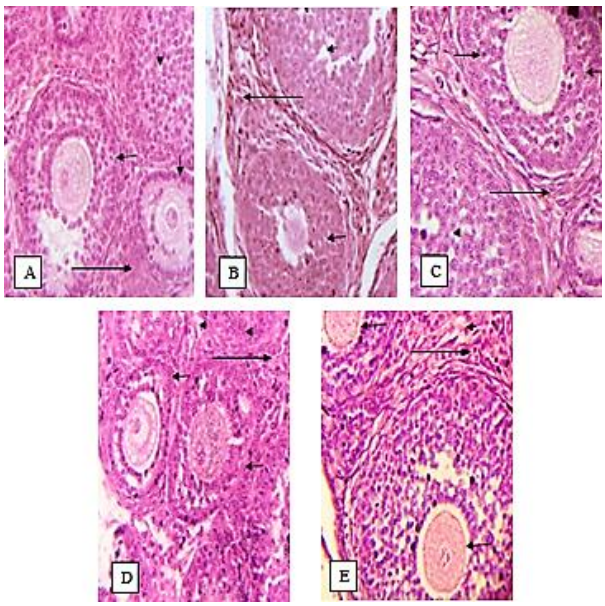

Figure 6: The photomicrographs of the immature female rat ovaries stained with H & E. ***Key:*** **A** – Control group with follicles (short arrow and arrowhead) and normal ovarian stroma (long arrow); **B** – BH extract 10 mg/kg, **C** – BH extract 100 mg/kg, **D** – BH extract 1000 mg/kg and **E** – Estradiol 10 mg/kg showing follicles (short arrow and arrowhead) and ovarian stroma (long arrow)

DISCUSSION

Plants are endowed with bio-active compounds, which are secondary metabolites with therapeutic and prophylactic properties. The knowledge of extract yield is important in the proper planning of experimental designs and helps to maximize time and adequate use of available funds [10]. In this study, the increase in body weight of immature rats in the BH extract treatment groups and estradiol group corresponded with an increase in daily feed consumption. Changes in body weight before and after treatments were significant ($p < 0.05$) in control and BH extract (10 mg/kg) groups. Thus, BH extract and estradiol at administered doses did not negatively affect appetite, digestion or nutrient absorption. This result contradicts Hirschberg's earlier report that estrogen decreased food intake [11].

On the other hand, the estradiol-treated group exhibited significantly ($p < 0.05$) increased uterine wet weight and estradiol blood levels. The BH extracts induced a non-statistically significant ($p > 0.05$) increase in blood estradiol levels and the uterine wet weight was not increased in the immature female rats. Extracts of the roots of *Carica papaya* and *Garcinia kola,* have been reported to possess different uterotrophic responses. The methanol extract of *Garcinia kola* seed was reported to significantly increase the uterine weight of immature albino rats [12]. However, chloroform extract of *Carica papaya* seed significantly decreased estradiol blood levels [13] and a decrease in uterine weight was reported in aqueous extract of *Carica papaya* seed [14].

The evidence that a test chemical induces uterotrophic (estrogenic) response is its ability to increase uterine weight caused by estrogen receptor-mediated fluid absorption into the uterus and cellular proliferation of uterine tissue. An estrogen-induced uterine weight has been reported previously [2,15]. The absence of an estrogenic effect in this study could be due to the bi-herbal extract not activating estrogen receptors and therefore not altering the uterine weight of the immature rats at the experimental doses.

Photomicrographs of hematoxylin and eosin-stained tissues from BH extract-treated groups showed normal uterine lining with small glands. The size of the endometrial glands compared with control and estradiol-treated rats could result from the BH extract not activating estrogen receptors in immature rats' uteri. The cervix and the ovaries showed normal cellular and structural presentations.

CONCLUSION

Aqueous bi-herbal extracts of *Garcinia kola* and *Carica papaya* root do not increase the blood estradiol levels and uterine weight in immature female rats. The results show that the bi-herbal composition is non-estrogenic and is unlikely to cause infertility and cancers associated with high estrogen levels in its use in females. However, clinical studies are required to validate this claim.

DECLARATIONS

Acknowledgements

Dr Anne Itemire is grateful to Mrs M Omuoarebun, the mother of my dear late friend Clara Omoarebun, who generously shared her knowledge with her, including the utilization of the bi-herbal (BH) root extract to effectively treat abnormal uterine bleeding.

Funding

None provided.

Ethical approval

Ethical approval (no. EC/FP/021/20) was obtained from the Ethics Committee on the Use of Animals for Experimental Procedures, Faculty of Pharmacy, University of Benin.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dr. Evi E. Bafor designed and supervised the study while Anne O. Itemire performed the experiments and wrote the manuscript.

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