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Effect of the phytoestrogen rich fraction of M*illettia aboensis* on carbohydrates metabolism and gastric emptying in animal model of menopause.

Daniel Lotanna Ajaghaku [1,*], Levi Onyebuchi Idokoja [1] and Paul Chinwuba [2]

[1] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria.*
[2] *Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, P.M.B 410001, Nsukka, Enugu State, Nigeria.*

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Abstract

This study investigated the effects of the phytoestrogen-rich fraction (PERF) of *Millettia aboensis* root extract on carbohydrate metabolism and gastric emptying in an ovariectomized rat model of menopause. Phytochemical analysis revealed that the ethyl acetate fraction had the highest phytoestrogen (1280 mg GE/g) and phenolic (900 mg GAE/g) content. In the carbohydrate metabolism assay, PERF at doses of 800 mg/kg and 1600 mg/kg significantly reduced blood glucose levels at 30 and 45 min post-administration compared to the control, suggesting potential inhibition of the α-glucosidase enzyme. PERF also exhibited prokinetic effects in the gastric emptying assay, with the 1600 mg/kg dose showing a higher percentage of gastric emptying than the 800 mg/kg dose. These findings indicate that PERF of *Millettia aboensis* root extract may be effective in managing metabolic disorders and constipation associated with menopause, possibly through mechanisms similar to acarbose and metoclopramide, respectively. This study highlights the potential of phytoestrogens as an alternative approach to address metabolic and gastrointestinal complications of menopause, warranting further investigation into their mechanisms of action and clinical applications.

keywords: Phytoestrogens; *Millettia aboensis*; Menopause; Carbohydrate Metabolism; Gastric Emptying; Metabolic Disorders; Ovariectomized Rat Model

1. Introduction

Menopause is not an illness, but can serve as a risk factor; it is a natural stage in a woman's life resulting from hormonal changes [1]. During menopause, the most prevalent metabolic issues include dyslipidemia, insulin resistance, hyperinsulinemia, type 2 diabetes, and impaired glucose tolerance [2], with a general prevalence of 30.1% [3]. As women approach the age of 60 years, they often encounter these chronic metabolic conditions, which affect both their quality of life and longevity. Consequently, the onset of menopause signals the need for various preventive measures to enhance the quality of life and extend the lifespan [4]. Menopause should be viewed as a time for heightened "diabetes vigilance" since many common symptoms experienced by women during this phase, such as significant weight changes, frequent urination, recurring urogenital infections, fatigue, weakness, irritability, blurred vision, excessive thirst, increased appetite, and sexual dysfunction, may be linked to carbohydrate disorders (non-insulin-dependent) rather than climacteric syndromes [2]. Glucose intolerance and hyperinsulinemia elevate the risk of cardiovascular disease in postmenopausal women who are thought to have diminished glucose tolerance. This age-related decline in glucose metabolism may be associated with postmenopausal hypoestrogenism. The connection between carbohydrate metabolism and circulating sex hormones has been suggested by variations in carbohydrate metabolism associated with different menstrual cycle phases [5]. Undiagnosed or untreated metabolic disorders are believed to negatively affect the duration and quality of life of postmenopausal women [2]. Although estrogen is commonly used in hormone

* Corresponding author: Daniel Lotanna Ajaghaku

replacement therapy (HRT), many researchers have noted its effectiveness in treating postmenopausal conditions while also highlighting adverse effects, such as an increased risk of ovarian cancer [6], irregular bleeding, migraines, and endometriosis [7]. The negative effects of synthetic estrogen have led to the development of natural therapeutic alternatives.

Historically, natural products have been crucial in the treatment and prevention of various diseases, as well as in drug discovery [8]. Phytoestrogens are plant-based polyphenolic compounds that exhibit estrogen-like biological effects [9]. These compounds are found in a diverse range of foods and offer numerous health benefits such as reducing the risk of osteoporosis, heart disease, breast cancer, and menopausal symptoms [10]. Phytoestrogens derived from plants are noted for their structural and functional resemblance to human estrogen, making them potentially beneficial in addressing postmenopausal conditions [11]. Thus, researchers are exploring the potential of phytoestrogens in disease treatment, with the aim of circumventing the negative side effects associated with synthetic estrogens and hoping for broader effectiveness. Anwuchaepe *et al*.[12] found that the root extracts of *Millettia aboensis* demonstrated significant in vivo estrogenic activity, with a high concentration of phytoestrogens, such as genistein and daidzein, in its ethyl acetate fraction. Subsequently, they confirmed the anti-osteoporotic properties of *Millettia aboensis*in an ovariectomized animal model of menopause [13]. In a review focusing on selective estrogen receptor modulators, SERMs (tamoxifen and raloxifene), Oseni *et al*. [10] highlighted various studies comparing phytoestrogens and SERMs. Although a certain Cochrane review database did not find clear evidence of phytoestrogens alleviating menopausal symptoms, numerous retrospective studies have demonstrated their significant role as an alternative treatment for menopausal symptoms, maintaining bone mineral density, and influencing breast cancer development, addressing recent concerns about the potential adverse effects of HRT. Phytoestrogens are commonly found in many natural dietary supplements and are widely promoted as natural alternatives to estrogen replacement therapy [14]. This study aimed to investigate the effectiveness of *Millettia aboensis* root extracts in managing metabolic disorders in postmenopausal women by assessing their effects on carbohydrate metabolism and gastric emptying.

2. Materials and methods

2.1. Collection and Identification of the Plant Material

The roots of *Millettia aboensis* were collected from the wild in Abagana, Anambra State, Nigeria, and their identity was confirmed by Nwafor Felix, a taxonomist in the Department of Pharmacognosy and Environmental Medicine at the University of Nigeria, Nsukka, Enugu State. After collection, the plant materials were cleaned, the roots were chopped into smaller pieces, and then left to air-dry at room temperature for three weeks before being ground into a powder using a mechanical grinder.

2.2. Animal source

For this study, female Wistar Albino rats weighing 100–250 g were used. The animals were sourced from the animal facility at the Department of Pharmacology, Enugu State University of Science and Technology, Agbani. They were kept under standard laboratory conditions, including a 12-hour light cycle, room temperature, and 40-60% relative humidity, and were provided with rodent feed. Rats had unrestricted access to food and water. All experiments involving animals adhered to NIH guidelines for the care and use of laboratory animals (National Institute of Health (NIH) (2011) Pub No: 85-23).

2.3. Ethical approval

Ethical approval was obtained, and all animal experiments were conducted in accordance with the guidelines of the Animal Ethics Committee of the Enugu State University of Science and Technology (Ethical Approval Number ESUT/AEC/2022/0155/AP098).

3. Reagents and equipment

3.1. Reagents

The chemicals and experimental reagents utilized included methanol (Guangdong Guanghua Chemical Factory Co., Ltd, China), n-hexane, ethyl acetate, butanol, tween-80, distilled/deionized water, 2% AlCl3, 5% glucose solution, phenol red (0.5 mg/ml), 0.1N NaOH, 2% w/v trichloroacetic acid, Folin-Ciocalteu's phenol reagent, 7.6% Na2CO3 solution, oxytet spray, ketamine, and xyaline. All solvents and reagents were of analytical grade and laboratory reagents were freshly prepared, including distilled water, when necessary.

3.2. Equipment/Material

Camry EK5350 Model electrical weighing balance from China, a glucometer, GX160 Delmar 5.5 HP mechanical grinding machine, a microscope, a stopwatch, a Stuart water bath from the UK, a UV-VIS Spectrophotometer (Model 732, China), a centrifuge, a fume cupboard, a refrigerator, Whatmann filter paper, a micro pipette, solvent bottles, a dropper, a Naugra stirring rod from India, conical flasks, ADARSH beakers from India, foil, chromic catgut and silk, a dissection kit, test tubes, a spatula, a retort stand with clamps, a separating funnel, measuring cylinders, sample bottles, a mortar and pestle, a porcelain crucible, a test tube rack, an axe, a hoe, a cutlass, a sack bag, muslin cloth, a stainless-steel basin, plastic and glass funnels, cotton wool, paper tapes, and syringes with cannula.

3.3. Extraction of the plant

The cold maceration method was used for extraction. A sample weighing 3 kg was placed in a clean, dry solvent bottle. Methanol (99.5%) was added to the sample at a 1:10 ratio. The bottles were sealed, and the mixture was thoroughly stirred and left at room temperature for 72 h with occasional vigorous shaking. Following maceration, the mixture was subjected to a two-step filtration process. Initially, it was passed through a muslin cloth, the filtrate was collected in a stainless-steel basin, and the residue on the cloth was pressed to extract additional filtrate. In the second filtration step, a funnel with a Whatman filter paper was used. The extract was poured through this setup, resulting in a clear filtrate. The extract was then divided into smaller portions in beakers and evaporated to dryness in a water bath set at 40°C within a fume cupboard. After drying, the extracts were weighed and stored in a refrigerator at 4°C.

3.4. Fractionation of the extract

The crude methanol extract was fractionated using the liquid–liquid partition method. This process involved three organic solvents, ethyl acetate, butanol, and n-hexane, which were chosen to separate the crude extract into distinct fractions based on polarity, starting with the least polar solvent (with the lowest eluting power) and progressing to the most polar solvent (with the highest eluting power) to enhance separation efficiency. The crude extract was dissolved in 200 ml of water and placed in a separation funnel. Then, 200 ml of n-hexane was added, the funnel was sealed, and the mixture was shaken vigorously to ensure thorough mixing. After allowing the mixture to settle for 20 min, it was separated into two immiscible layers. The lower aqueous layer was drained and collected in a beaker, followed by the upper n-hexane layer, which was collected separately. This procedure was repeated twice with 200 ml of n-hexane each time, and by the third separation, the n-hexane layer was clear, indicating that nearly all n-hexane-soluble components were extracted from the crude extract. The process was then repeated using ethyl acetate, which is a more polar solvent. After the third separation with 200 ml of ethyl acetate, the ethyl acetate layer was clear, indicating that all ethyl acetate-soluble components were extracted. The final solvent used was butanol, which was the most polar of the three solvents. The process was repeated using 300 ml of butanol, and by the second separation, the butanol layer was clear, indicating that the first separation effectively extracted the remaining components from the crude extract. The solvent fractions, including the water fraction, were evaporated to dryness, weighed, and stored in a refrigerator at 4°C until further analysis.

3.5. Quantification of phytoestrogen content using genistein as standard

The method outlined by Cesar *et al*. [15] was employed to measure phytoestrogen levels in the fractions. Each fraction (100 µg/ml) was combined with 1 ml of 2% $AlCl_3$ dissolved in methanol, and this was done in duplicate. Absorbance readings were taken at 382 nm, ten minutes after the addition of $AlCl_3$. Blank solutions of the standard and samples containing only methanol, without $AlCl_3$, were prepared. Phytoestrogen levels were determined using the genistein calibration curve and reported as milligrams of genistein equivalent (GE) per gram of the extracts.

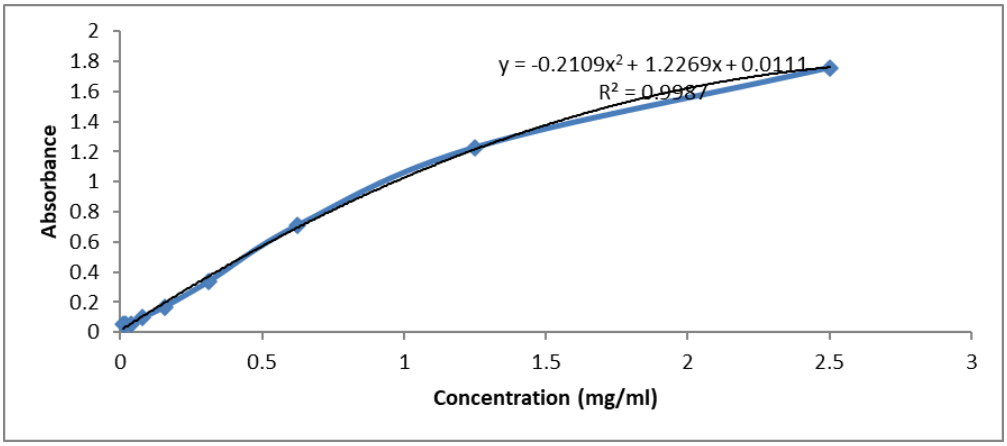

Figure 1 Genistein calibration curve

3.6. Quantification of total phenolic content using gallic acid as standard

The method outlined by Kim *et al*. [16] was used to assess the total phenolic content of the fractions. First, 1 mL of the extract at a concentration of 100 µg/ml was combined with 0.2 ml–Folin-Ciocalteu's phenol reagent. After a 5-minute interval, 1 ml of 7.6% Na2CO3 solution was added, followed by 2 ml of distilled water. This mixture, prepared in duplicate, was incubated at 40°C for 30 min. Subsequently, absorbance was measured at 760 nm using a UV-VIS spectrophotometer (Model 752, China), with a blank sample containing all components except the extract serving as a reference. The total phenolic content was calculated from a calibration curve created using gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

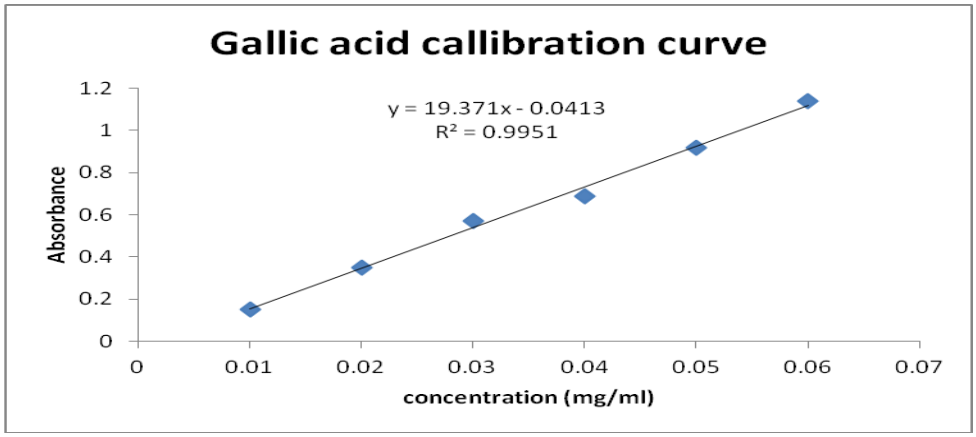

Figure 2 Gallic acid calibration curve

3.7. Induction of menopause

Following the method outlined by Shailajan *et al*. [17], vaginal cytology was performed on all animals to verify the regular estrous cycles. Every morning between 8 am and 10 am, vaginal smears were collected and examined under a microscope, observing two consecutive 4-day estrous cycles. Subsequently, the animals underwent ovariectomy (OVX) to induce menopause and to study reproductive changes after extract supplementation. Post-ovariectomy and vaginal smears from all mice were analyzed microscopically to confirm complete ovary removal, as indicated by the absence of epithelial cell cornification (diestrus phase). Thirty Wistar Albino rats, weighing between 100 and 250 g, were divided into six groups, each containing five animals. All groups except Group 6, which served as the sham-operated control, underwent ovariectomy. In the sham-operated control group (without OVX), the ovaries were exposed and gently handled, but not removed. The remaining animals underwent bilateral OVX through a dorso-lateral approach, involving a small lateral vertical skin incision with a surgical blade, followed by ligation and removal of the ovaries along the upper horn under general anesthesia with ketamine and xylazine (80 and 10 mg/kg body weight, respectively). The incisions were closed using suture materials (chromic catgut and silk) and treated with an oxytet spray to promote rapid wound healing. Precautions were taken to prevent infection throughout the OVX procedure. The animals were given a three-week recovery period before dosing, during which they were monitored for unusual behavior or side effects.

3.8. Determination of the effect of the phytoestrogen rich fraction on carbohydrates metabolism

Following a 12-hour fasting period, ovariectomized animals were given 100 mg/ml of corn starch (a carbohydrate) at a dosage of 3 g/kg. Subsequently, 250 mg/ml of the phytoestrogen-rich fraction (PERF) of *Millettia aboensis* orally administered to the animals. Groups 1, 2, and 3 received doses of 400 mg/kg, 800 mg/kg, and 1600 mg/kg of PERF, respectively. Group 4, serving as the negative control, was administered 5 ml of distilled water, whereas group 5, the positive control, received 50 mg/kg of acarbose. At the start of the experiment, fasting blood sugar (FBS) levels of the animals were measured and documented. The animals were challenged with commercial corn starch. Blood glucose levels were subsequently monitored at 15, 30, 45, and 60 min to assess the effect of phytoestrogens on carbohydrate metabolism.

3.9. Determination of the effect of the phytoestrogen rich fraction on gastric emptying

The procedure outlined by Scarpignato *et al*. [18] was used in this study, involving the animals mentioned in Section 2.9, which were divided into five groups. A test meal, which included a non-absorbable marker (0.5 mg/ml phenol red in 5% glucose solution), was administered to the animals via gavage. Group 1 was administered 5 ml of distilled water along with the test meal, whereas groups 2 and 3 received 800 mg/kg and 1600 mg/kg of PERF, respectively, with the test meal. Group 4 was administered 20 mg/kg metoclopramide with the test meal, and group 5 received 40 mg/kg tramadol with the test meal.

The animals in Group 1 were euthanized immediately following the administration of the test meal to establish the standard stomach concentration of phenol red, whereas the animals in the other groups were sacrificed 60 min later. The stomachs were accessed via laparotomy, swiftly clamped at both the pylorus and cardiac ends, and then removed. They were placed in 10 ml of 0.1N NaOH, cut into small pieces, and homogenized for 30 s. The resulting suspensions were left to settle for 20 min at room temperature, after which 5 ml of the supernatant was centrifuged for 10 minutes at 3000 rpm. Proteins in 4 ml of the supernatant were precipitated using 0.4 ml of trichloroacetic acid (20% w/v) and centrifuged again for 10 minutes at 6000 rpm. Following centrifugation, each supernatant was diluted tenfold with 0.1N NaOH. The absorbance of each sample was measured at 560 nm using a spectrometer. The percentage of gastric emptying (% GE) in each animal was determined using the following formula:

$$\%GE = \left(\frac{AP - CP}{AP}\right) X100$$

In this context, AP represents the absorbance of phenol red in the standard stomach, whereas CP denotes the absorbance of phenol red retrieved from the test stomachs. The rats that were euthanized immediately following the administration of the test meal (group 1) were used to establish standard stomachs, which contained 100% phenol red.

4. Results

4.1. Percentage Yield of Extract and Fractions

Table 1 displays the methanol extractive yield from the pulverized roots of *Millettia aboensis* and its fractions with various solvent polarities. A total of 163.8 g of extract was obtained from 3 kg of dried pulverized roots, equating to a yield of 5.46%. When this extract was further separated using solvents of different polarities, the water fraction produced the highest yield (39.07%), whereas the butanol fraction resulted in the lowest yield of 9.16%.

Table 1 Percentage yield of the extract and fractions

Fraction	Weight(g)	Percentage yield (%)
Crude extract	163.80	5.46
Water	64	39.07
Ethyl acetate	35	21.37
n-Hexane	20	12.21
Butanol	15	9.16

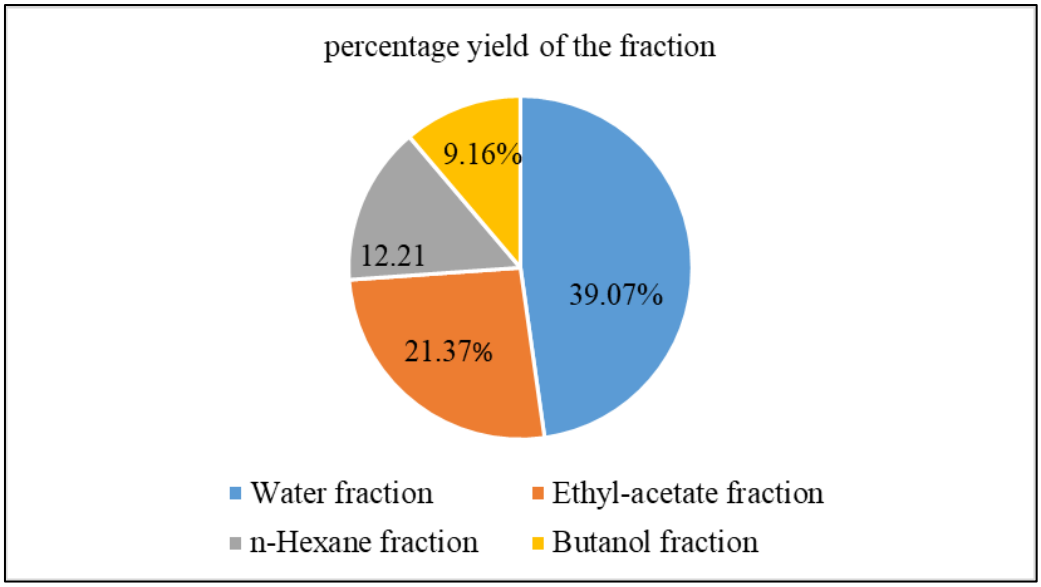

Figure 3 Percentage yield of the fractions

4.2. Phytoestrogen Content of the Fractions

Figure 4 illustrates the phytoestrogen levels in different fractions. Using a standard genistein calibration curve, the phytoestrogen content was measured and expressed as milligrams of genistein equivalent (GE) per gram. The results showed that the ethyl acetate fraction, known as the phytoestrogen-rich fraction (PERF), contained the highest phytoestrogen level at 1280 mg GE per gram of extract. In contrast, the butanol fraction had the lowest phytoestrogen content, with only 200 mg GE per gram.

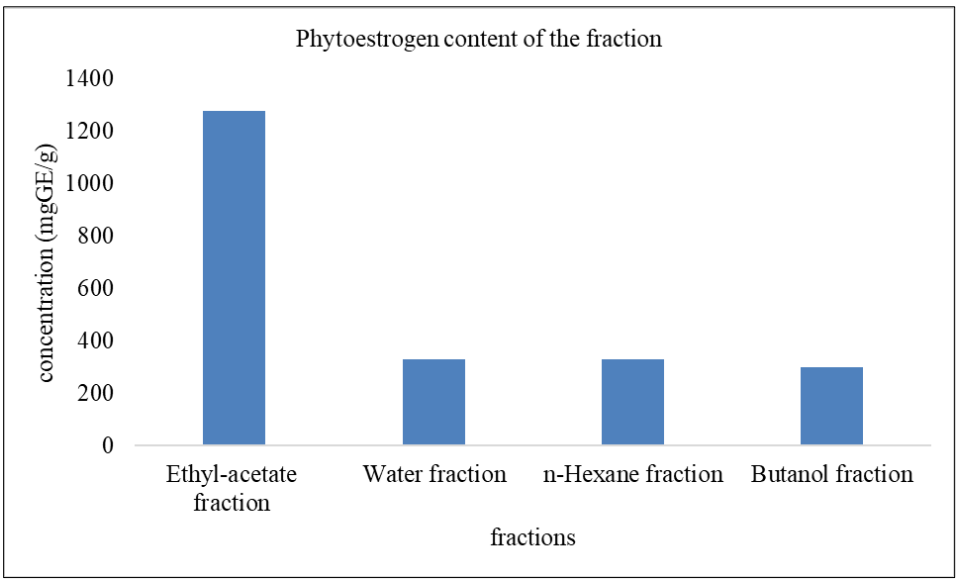

Figure 4 Phytoestrogen content of the fraction

4.3. Total Phenolic Content of the Fractions

Figure 5 shows the total phenolic contents of the fractions. Using a standard garlic acid calibration curve, the phenolic content was measured and expressed as milligrams of gallic acid equivalent (GAE) per gram. The results showed that the ethyl acetate fraction had the highest phenolic content at 0.900 mg GAE/g of extract, whereas the butanol fraction exhibited the lowest phenolic content at 470 mg GAE/g.

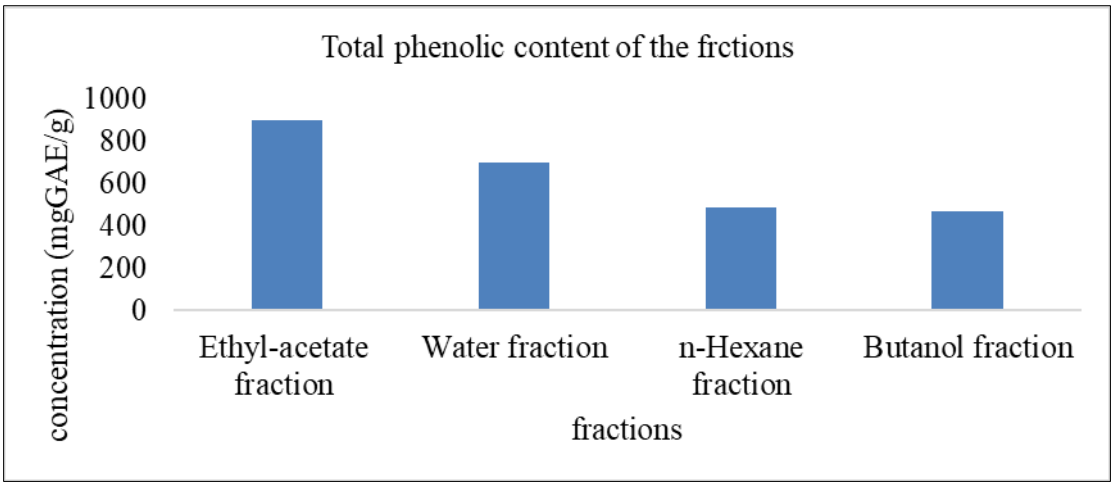

Figure 5 Total phenolic content of the fractions

4.4. Carbohydrate Metabolism Assay

Fig 6 presents a histogram illustrating the impact of varying doses of PERF from *Millettia aboensis* extract at intervals of 0, 15, 30, 45, and 60 min. Notably, there were significant decreases in blood glucose levels at the 30- and 45-minute marks for both the 800 mg/kg and 1600 mg/kg doses compared with the control group. Fig 7 depicts the different reductions in blood glucose levels in the treatment groups, as measured by their respective AUCs. Finally, Fig 8 is a graph that demonstrates the changes in blood glucose levels across the various treatments over the specified time intervals.

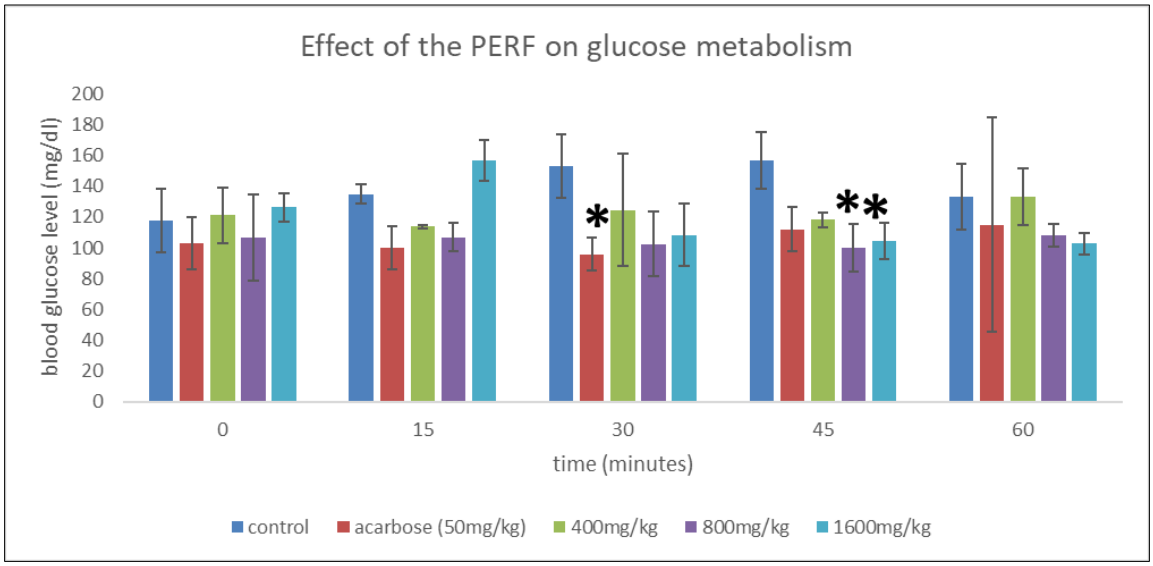

Figure 6 Effect of the PERF on glucose metabolism.

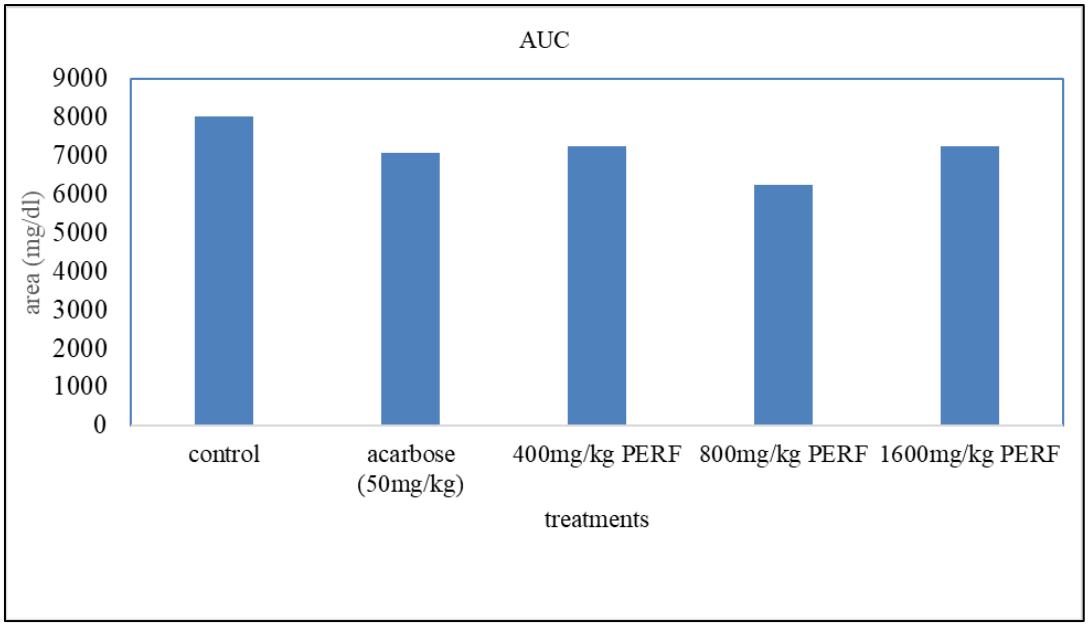

Figure 7 AUC of the different treatments

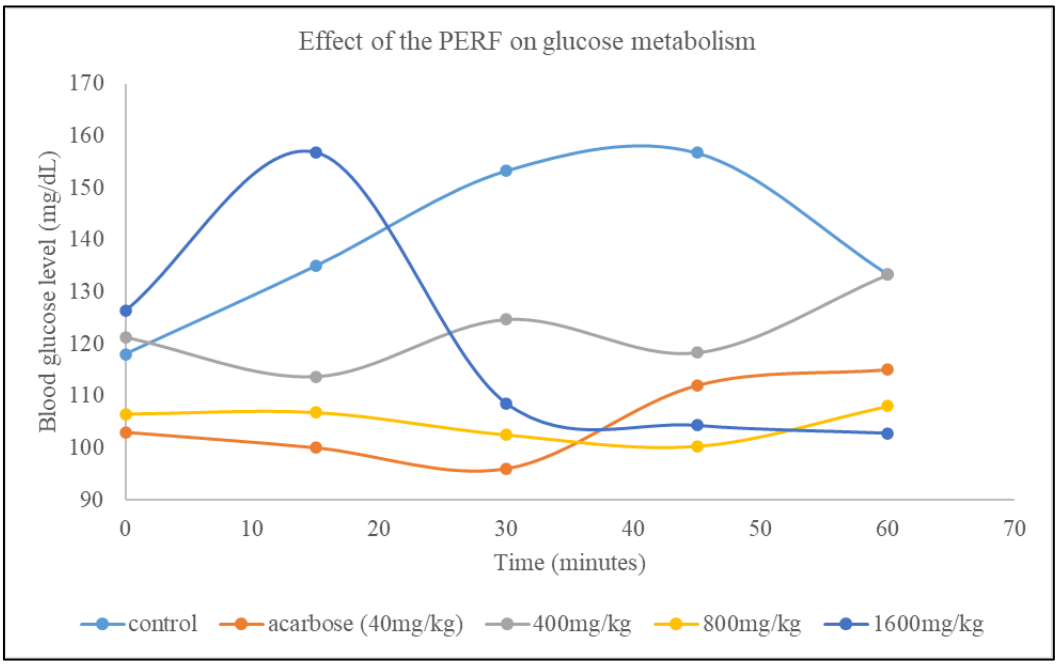

Figure 8 Effect of the PERF on glucose metabolism.

4.5. Gastric Emptying Assay

Fig 9 presents a histogram illustrating the impact of various doses of PERF on gastric emptying, compared to the standards metoclopramide (a prokinetic agent) and tramadol (an inhibitor of gastric emptying). These data indicate that PERF at doses of 800 mg/kg and 1600 mg/kg mirrors the effect of metoclopramide by enhancing gastric emptying. As the dose of the extract increased, as did the percentage of gastric emptying, the 1600 mg/kg dose of PERF resulted in a higher percentage of gastric emptying than the 800 mg/kg dose.

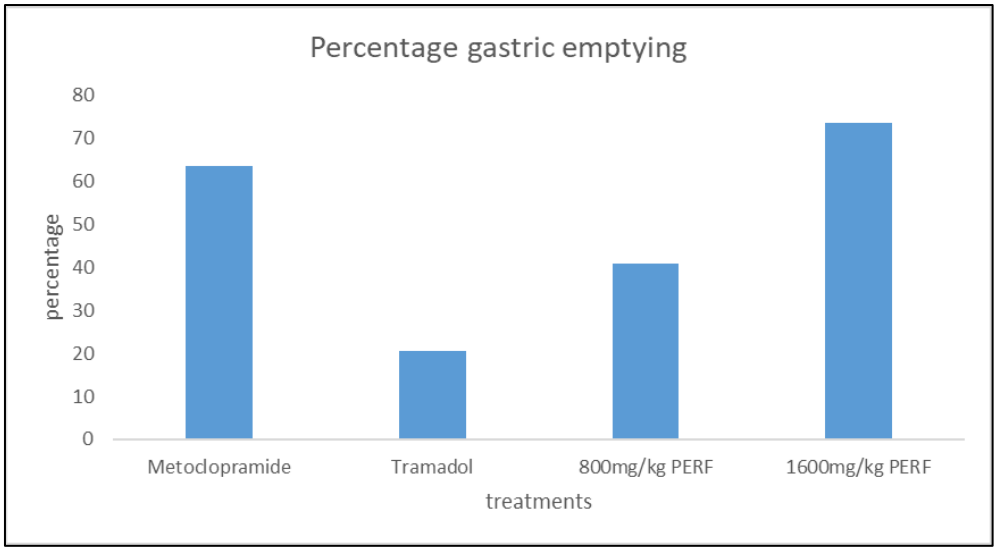

Figure 9 Effect of the PERF on gastric emptying

5. Discussion

The methanol extract yields from the pulverized roots of *Millettia aboensis* and its various solvent polarity fractions are presented in Table 1. From 3 kg of dried, pulverized roots, 163.8 g of extract was obtained, equating to 5.46% yield. When this extract was further divided using solvents of different polarities, the intermediate polar solvent ethyl acetate produced the highest yield at 39.07%, while butanol yielded the lowest yield at 9.16%. As shown in Fig 4, phytochemical analysis of these fractions revealed that the ethyl acetate fraction was rich in phytoestrogens, containing a total of 1280 mg GE/g, whereas the butanol fraction had the lowest phytoestrogen content at 200 mg GE/g. Additionally, the ethyl acetate fraction had the highest phenolic content (900 mg GAE/g), whereas the butanol fraction had the lowest content (470 mg GAE/g) (Fig 5). Increasing evidence suggests that consuming certain plants or their phytoestrogen-rich compounds can be an effective tool for preventing and treating various diseases related to aging, metabolism, and menopausal symptoms [19,20,21]. These properties suggest that they could be valuable in addressing the metabolic issues associated with menopause.

Phytoestrogenic substances such as soy genistein have been suggested as promising agents for enhancing metabolism and treating metabolic disorders [22]. In this study, PERF at doses of 800 mg/kg and 1600 mg/kg showed a significant reduction in blood glucose levels at 30- and 45-minute intervals (Figs 6-8). Similar to acarbose, the standard drug used in this study, one approach to managing glucose metabolic disorders is through the inhibition of the alpha-glucosidase enzyme. The observed decrease in blood glucose levels, akin to the effect of acarbose, indicates that PERF may also inhibit alpha-glucosidase. Sphincter tone and perianal surgery are the primary factors contributing to the high incidence of constipation in postmenopausal women, with excessive straining during defecation being the most prevalent symptom [23]. As illustrated in Fig 9, PERF demonstrated effectiveness as a prokinetic, with the 800 mg/kg and 1600 mg/kg doses of the extract showing a significant percentage of gastric emptying compared to the controls.

6. Conclusion

The current study indicates that the ethyl acetate portion of *Millettia aboensis* is a fraction of the root extract that is rich in phytoestrogens. This PERF demonstrated notable effects on carbohydrate metabolism and gastric emptying, suggesting its potential use in addressing metabolic disorders and constipation associated with menopause.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no competing interests

Statement of ethical approval

Ethical approval was obtained from the Animal Ethics Committee of Enugu State University of Science and Technology.

Data availability

The materials used in this study are described in the References section. In addition, any other information related to this work will be provided upon reasonable request by the corresponding author.

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