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Combination effect of phytoestrogen and phenol rich fractions of *Ochna schweinfurthianaon* gastric emptying in ovariectomized animal model of menopause.

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Abstract

The present study investigated the combined effects of phytoestrogen and phenol-rich fractions of *Ochna schweinfurthianaon* gastric emptying in an ovariectomized animal model of menopause. Gastroparesis, a chronic gastrointestinal disorder characterized by delayed gastric emptying, is exacerbated by a decline in estrogen levels during menopause. The bark of *Ochna schweinfurthiana*was extracted and the extract was fractionated using various solvents. The total phenolic and phytoestrogen contents of the extract and fractions were quantified using the Folin Ciocalteu's assay and a genistein calibration curve, respectively. Menopause was induced in female Swiss albino rats by ovariectomy. The effects of additive (PE 132:PH 251 mg/kg) and synergistic (PE 160:PH 130 mg/kg) combinations of phytoestrogen and phenol-rich fractions on gastric emptying were evaluated using a phenol red meal. Metoclopramide and tramadol served as the positive and negative controls, respectively. The rate of gastric emptying, time required for 50% gastric emptying, and stomach contents were assessed. The results showed that the combination doses exhibited a prokinetic effect, increasing the gastric emptying rate and reducing the time required for 50% gastric emptying, similar to metoclopramide. The stimulatory effect of the phytoestrogen and phenol-rich fractions may be mediated through mechanisms other than the classical estrogen receptor signaling pathway. In conclusion, the combination of phytoestrogen and phenol-rich fractions from *Ochna schweinfurthiana* demonstrated a prokinetic effect on gastric emptying in an ovariectomized animal model of menopause, suggesting their potential as an alternative therapeutic strategy for gastroparesis in postmenopausal women.

Keywords: Gastroparesis; Menopause; Phenols; Phytoestrogens; *Ochna schweinfurthiana*; Gastric Emptying; Ovariectomized

1. Introduction

Gastroparesis is a chronic disorder characterized by delayed gastric emptying in the absence of mechanical obstruction [1]. It significantly impairs the normal motility of the stomach, leading to a variety of distressing symptoms such as nausea, vomiting, early satiety, bloating, and abdominal pain. The etiology of delayed gastric emptying is diverse, with the most common forms including idiopathic, medication, diabetic (types 1 and 2), and post-surgical complications. Emerging evidence has also shown that hormonal fluctuations, particularly those occurring during menopause, play a role in the onset and exacerbation of delayed gastric emptying [2]. Gastric motility is largely regulated by the enteric nervous system, primarily consisting of excitatory acetylcholine, inhibitory NO, and vasoactive intestinal peptide

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effectors [3]. The importance of NO in gastric function, produced in this case by neuronal nitric oxide synthase (nNOS), has been established by findings of pyloric hypertrophy and gastric dilation in nNOS (-/-) mice [4]. The catalytic activity of NOS depends on a dimerization step, facilitated by (6R)-tetrahydrobiopterin (BH4). BH4 is an endogenously synthesized molecule and a cofactor for all NOS enzymes (nNOS, eNOS, and iNOS) [2]. Diabetes induction by streptozotocin significantly impairs these parameters and leads to delayed gastric emptying in female rats [5]. Female rats are more dependent on nitrergic regulation of gastric function than males, and this may have clinical implications in patients. Thus, it is possible that females are more vulnerable to gastroparesis because of changes in the BH4-nNOS pathway secondary to changes in female hormones. Although conflicting reports exist, evidence suggests that elevated levels of circulatory estradiol-17b (E2), but not progesterone, regulate gastric emptying in healthy females [6]. These studies further indicated that E2 elevates NO levels and regulates gastric motility. FORKO mice lacking the follicle-stimulating hormone receptor (FSH-R) exhibit profound changes in the ovarian structure and secondary sex organs. FSH-R gene disruption causes complete loss of ovarian estrogen production (95 %). However, circulatory progesterone levels were decreased by 70 % in FSH-R null mice. Chronic deficiency of circulating estrogen in these mice also causes important metabolic alterations that can induce obesity with aging, a well-known detrimental factor for the development of type 2 diabetes in humans [7]. Estrogen replacement therapy promptly induced uterine growth and reversed adipose tissue accumulation, suggesting a functional role for estrogen in these mice [7]. Reduced levels of E2 have been reported to downregulate BH4 biosynthesis and, therefore, impair nNOS-mediated gastric motility in the FORKO mouse model of chronic estrogen deficiency compared to WT SVE129 female mice. The importance of estrogens in BH4 synthesis comes from the fact that E2 treatment elevated the expression of both GCH-1 and BH4 in rat brain neurons via an estrogen receptor-mediated event [8]. A previous report using in vitro hyperglycemic conditions suggested that E2 supplementation restored the impairment of both BH4 biosynthesis and NO generation via ERα in bovine aortic endothelial cell cultures [9]. Estrogen supplementation has also been shown to attenuate DN progression of diabetic nephropathy in diabetic rats [10]. Most importantly, Shah et al. [11] found that nNOS protein expression and nitrergic relaxation in the gastric fundus were increased in estrogen-but not in progesterone-treated ovariectomized mice. Together, these data collectively indicate that a reduction in E2 levels can lead to reduced nitrergic relaxation and delayed gastric emptying (gastroparesis).

Menopause is marked by a decline in estrogen, a hormone that plays a critical role in maintaining gastrointestinal function [12]. Loss of estrogen during menopause impairs gastric motility and increases sensitivity to gastric distention, potentially exacerbating or even triggering gastroparesi-like symptoms in predisposed women [2]. Given the limitations of conventional hormone replacement therapy and other treatments for gastroparesis, including dietary modifications, prokinetic agents, and antiemetics, alternative therapeutic strategies have been explored [13]. One such promising approach involves the use of phytocompounds, such as phytoestrogens, which are naturally occurring plant compounds that structurally and functionally resemble human estrogen and phenolic compounds. Our previous study indicated that compounds from *Ochna schweinfurthiana* provides benefits for menopausal symptoms and bone density, but their role in gastrointestinal disorders remains largely unexplored [14]. This study investigated the combined effects of phytoestrogen- and phenolic-rich fractions of *Ochna schweinfurthiana* on gastric emptying in an ovariectomized animal model of menopause.

2. Materials and Method

2.1. Plant Materials

The bark of *Ochna schweinfurthiana*was collected from Agulu in Anambra State, Nigeria, and authenticated by a trained taxonomist, Mr. Felix Nwafor, Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria. A voucher specimen (no. PCG 521/A/043) was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Agulu, for future reference. The plant materials were subsequently air-dried at room temperature and pulverized using a mechanical grinding machine (GX160 Delmar 5.5HP).

2.2. Animal Source

Female Swiss albino rats were used in this study. All the animals were obtained from the Animal House of the Department of Pharmacology, Enugu State University of Science and Technology. The animals were housed under standard laboratory conditions of 12 h light, room temperature, 40-60% relative humidity, and fed with rodent feed (Guinea Feeds Nigeria Ltd.). Animals were allowed free access to food and water. The maintenance and care of all animals were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH 86-123 strictly adhered to. Ethical approval was obtained from the

Animal Ethical Committee of the Enugu State University of Science and Technology (Approval number: ESUT/AEC/0169/AP204) where the animal studies were conducted.

2.3. Extraction and Fractionation

*Ochna schweinfurthiana*bark powder was macerated in methanol for 72 h with intermittent shaking. The resulting solution was filtered using Whatman filter paper, and the filtrate was concentrated in vacuo using a rotary evaporator (RE300 Model, United Kingdom) at 40°C. Two-thirds of the extract was subjected to liquid-liquid partition successively with n-hexane, ethyl acetate, and butanol using a 1 L separating funnel. The fractions were concentrated in vacuo using a rotary evaporator at 40°C to obtain the n-hexane-soluble, ethyl acetate-soluble, butanol-soluble, and water-soluble fractions. The extracts and all fractions were stored in a refrigerator at 0-4°C for further use.

2.4. Total Phenolic Content of the Extracts and Fractions by Folin Ciocalteu's Assay

The total phenolic content of the extract and fractions was determined using the method described by Ajaghaku et al. [15]. One milliliter of the extract (100 µg/ml) was mixed with 0.2 ml–Folin-Ciocalteu's phenol reagent. After 5 min, 1 ml of 7.6% Na2CO3 solution was added to the mixture, followed by the addition of 2 ml of distilled water. The mixture (in duplicate) was incubated at 40°C for 30 min, after which the absorbance was measured at 760 nm using a UV-VIS spectrophotometer (Model 752, China). The total phenolic contents were estimated from the calibrated curve, which was prepared from gallic acid solution and expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract.

3. Quantification of phytoestrogen content using genistein as standard

The method outlined by Ajaghaku et al. [16] was employed to quantify phytoestrogen levels in the fractions. Each extract and fraction, at a concentration of 100 ug/ml, was combined with 1 ml of 2% AlCl3 dissolved in methanol, and this was done in duplicate. The absorbance was measured at 382 nm ten minutes after the addition of AlCl3. Blank solutions consisting of the standard and samples without AlCl3 (methanol only) were also prepared. Phytoestrogen content was calculated using a genistein calibration curve, as described below, and expressed in milligrams of genistein equivalent (GE) per gram of extract or fraction. Various concentrations of genistein (2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0391, 0.0195, and 0.0098 mg/ml) were prepared in duplicate following the same procedure. The genistein calibration curve was obtained by plotting the absorbance against concentration.

3.1. Induction of menopause

To confirm regular estrus cycles, vaginal cytology was performed on all animals. Each morning between 8 am and 10 am, vaginal smears were examined under a microscope to identify at least two consecutive 4-day estrus cycles. The animals underwent ovariectomy (OVX) to induce menopause and study reproductive changes after supplementation with phenol/phytoestrogen-rich extract. Post-ovariectomy, vaginal smears were again examined microscopically to confirm the complete removal of ovaries, as indicated by the absence of epithelial cell cornification (Diestrus phase). All groups of animals, except those that served as sham-operated controls, underwent OVX. In the sham-operated controls, the ovaries were exposed and gently manipulated but not removed. The remaining animals underwent bilateral OVX through a dorso-lateral approach, involving a small lateral vertical skin incision made 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, i. m.). The incisions were closed using suture materials (chromic catgut and silk) and treated with an oxytet spray to promote quick wound healing. Precautions were taken to prevent infection throughout the OVX procedure. The animals were given a 3-week recovery period before dosing, during which they were monitored for unusual behavior or side effects.

3.2. Dose selection for combination study

An initial unpublished investigation into the estrogenic dose-response effects of phenol- and phytoestrogen-rich fractions from *Ochna schweinfurthiana*, as well as the interaction of different dose ratio combinations, indicated that when phytoestrogen (PE)-and phenol (PH) rich fractions were combined in dose ratios of PE 132:PH 251 mg/kg and PE 160:PH 130 mg/kg resulted in additive and synergistic interactions, respectively.

3.3. Determination of the effect of combinations of phytoestrogen and phenolic rich fractions of *Ochna schweinfurthiana on* gastric emptying

This study employed a modified version of the method outlined by Mbagwu et al. [17]. Animals were administered a test meal containing a non-absorbable marker (0.5 mg/ml phenol red in a 5% glucose solution) via gavage. Group 1 was

administered 10 ml/kg of 5% Tween 20 along with the test meal. Groups 2 and 3 received an additive combination dose (PE 132:PH 251 mg/kg) and a synergic combination dose (PE 160:PH 130 mg/kg) of phytoestrogen and phenol-rich fractions, respectively, with the test meal. Group 4 was administered 20 mg/kg metoclopramide, and group 5 received 40 mg/kg tramadol, both with the test meal.

The animals in group 1 were euthanized immediately after the test meal to establish the standard stomach concentration of phenol red, while those in the other groups were sacrificed 60 min later. The stomachs were accessed via laparotomy, quickly clamped at 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 mixtures were allowed to settle for 20 min at room temperature, and 5 ml of the supernatant was centrifuged for 10 min 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 calculated using the following formula:

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

where AP = Absorbance of phenol red in the standard stomach and CP is the absorbance of phenol red recovered from the test stomachs. Rats sacrificed immediately after test meal administration (group 1) were used to establish the standard stomachs (100% phenol red in the stomach). The gastric emptying rate was calculated as the quotient of change in gastric emptying by change in time, while it took to empty half the gastric content was derived from regression analysis of the plot of percentage gastric content vs. time.

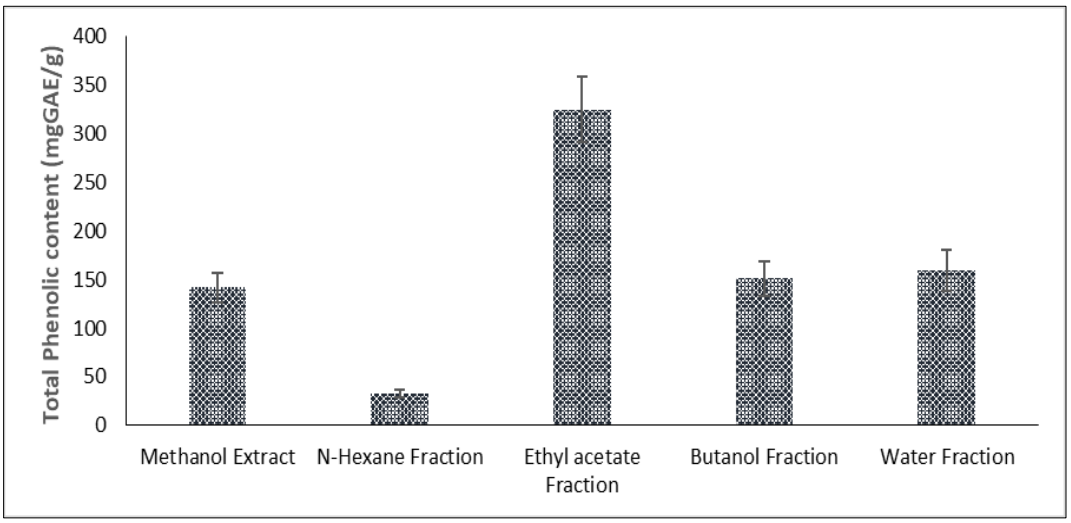
3.4. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA, followed by Newmann-Keuls multiple range tests. Statistical evaluations were conducted using GraphPad version 3.1, and P values were deemed statistically significant when $P< 0.05$

4. Results

4.1. Total phenolic content

The total phenolic content in the extract and its fractions was determined using a gallic acid calibration curve and expressed as milligrams of gallic acid equivalent per gram of extract or fraction (Figure 1). The extract contained a significant number of total phenols (142.16 mgGAE/g), with the majority being concentrated in the ethyl acetate fraction, which is known for its high phenol content, while the n-hexane fraction had the lowest phenolic content.



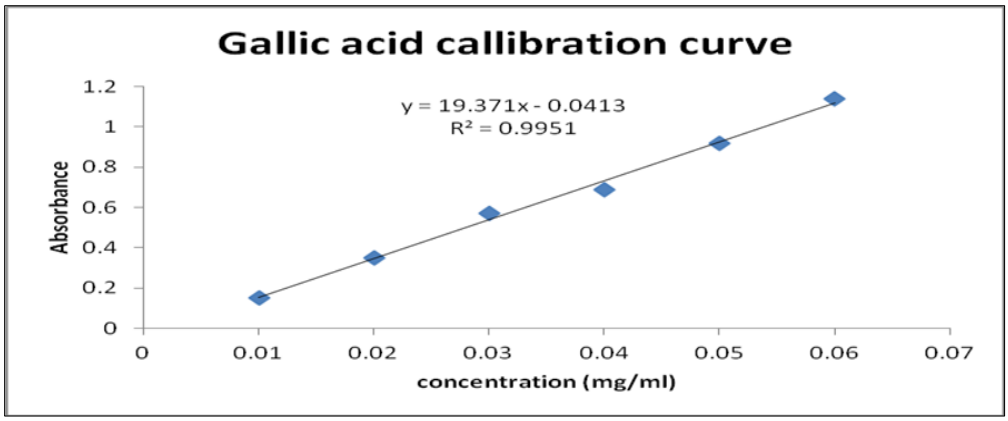


Figure 1 Total phenolic content of the extract and fractions of *Ochna schweinfurthiana*

4.2 Phytoestrogen content

The extract revealed a phytoestrogen concentration of 1680 mg genistein Eq/g (Figure 2). Through liquid-liquid partitioning, the phytoestrogen compounds in the extract were concentrated in the water fraction. When compared to the other fractions, the phytoestrogens in the water fraction showed more than a tenfold difference. The phytoestrogen content in the fractions increased as solvent polarity increased.

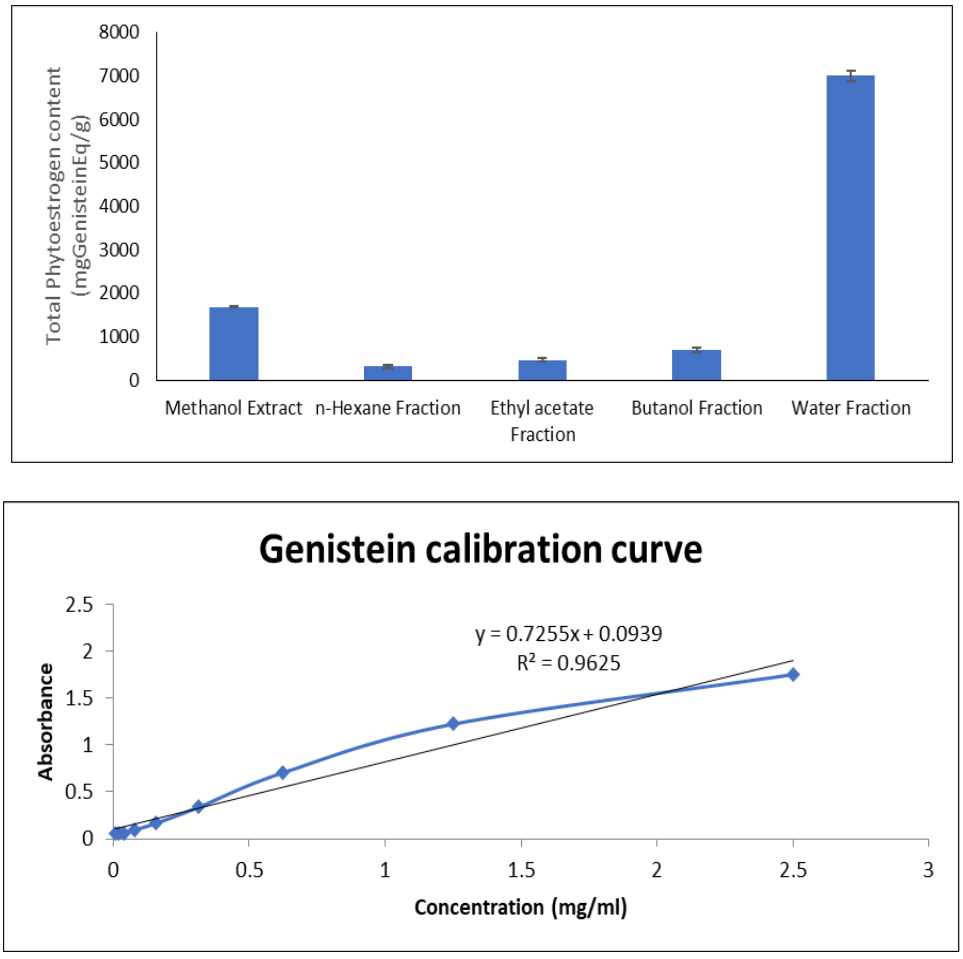
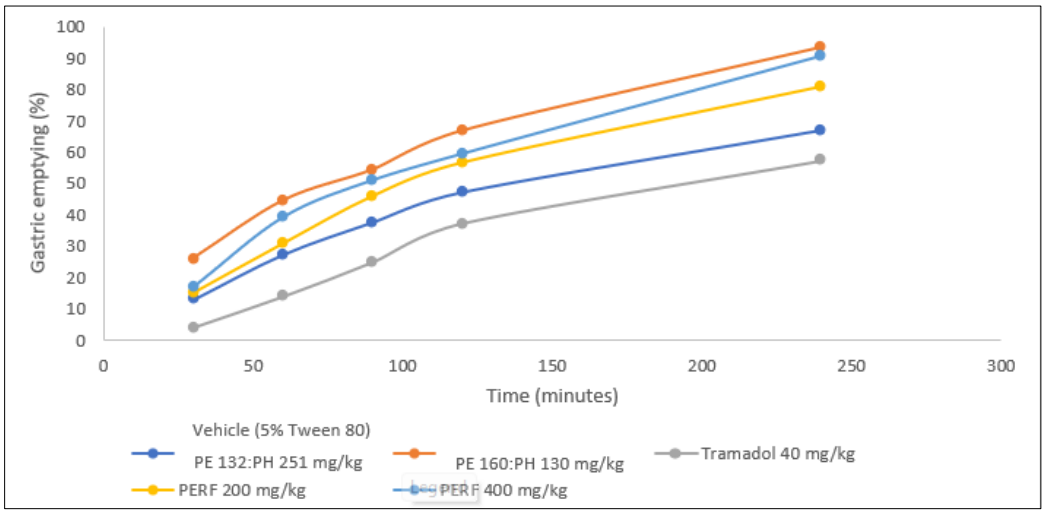


Figure 2 Total phytoestrogen content of the extract and fractions of *Ochna schweinfurthiana*

4.3 Gastric emptying

Figure 3 illustrates the percentage of gastric emptying and stomach contents after time-dependent gastric emptying. An increase in gastric emptying corresponded to a decrease in the stomach content. The increase in gastric emptying observed for tramadol was lower than that in the vehicle control group, indicating a reduction in gastric emptying compared to the vehicle control group. Treatment with combined doses of phytoestrogen and phenol-rich fractions at both doses resulted in gastric emptying above the vehicle control curve, similar to metoclopramide. The rate of gastric content emptying showed that tramadol, unlike other treatments, had a slower rate of 0.647 µmol phenol red/min than the vehicle control (0.908 µmol/min) (figure 4). The combination of phytoestrogen and phenol-rich fractions exhibited a prokinetic effect, increasing the gastric content emptying rate akin to metoclopramide. The time required to empty half of the gastric contents is shown in figure 3. Time-dependent percentage gastric emptying revealed that the vehicle control took 134.16 minutes to empty half of its gastric content (figure 5a), while tramadol required 197.85 minutes for the same effect (Figure 5b). The prokinetic effect of metoclopramide was confirmed by the shorter time of 84.43 minutes needed to empty half of the stomach content in that group (figure 3c), which is comparable to the values recorded for combination doses at PE 132:PH 251 mg/kg (97.51 min) and PE 160: PH 130 mg/kg (82.27 min), as shown in figures 3d and 3e, respectively. Multiple comparison analyses indicated that time-dependent gastric emptying occurred in all groups from 30 to 240 min of monitoring (figure 6).



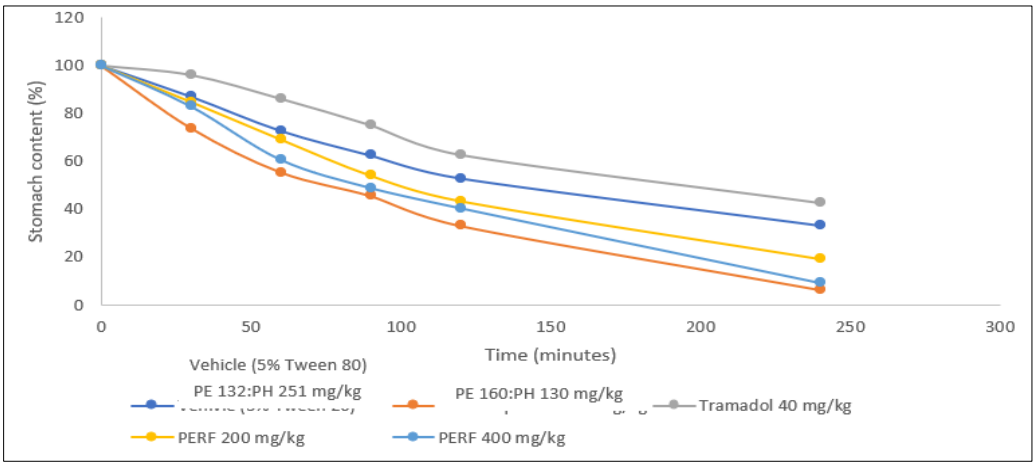


Figure 3 Effect of Phytoestrogen rich fraction on gastric emptying

A significant ($P < 0.05$) reduction in stomach content was observed compared to the content at 0 min, except for the tramadol-treated groups, which showed no significant ($P > 0.05$) reduction 30 min after phenol red administration. Compared to the other treatment and control groups, tramadol showed significantly ($P < 0.05$) higher stomach contents from 30 to 240 min. Conversely, metoclopramide exhibited a lower stomach content than the other treatment and

control groups. This reduction in stomach content was significant (P<0.05) across the groups from 30 to 240 min, except for the PE 160:130 mg/kg combination dose-treated group, which showed similar stomach contents at 60-, 90-, and 240-min intervals. Treatment with the combination doses at both tested doses did not produce a significant (P>0.05) increase in gastric emptying 30 min after phenol red administration; however, the increase in gastric emptying produced by the PE 160:130 mg/kg combination dose became significant from 60 min, whereas that of the PE 132:251 mg/kg combination dose was recorded from 90 min after phenol red administration. The effects produced by both combination doses were similar at 30-, 90-, and 120-minutes intervals with no significant (P>0.05) differences. Significant (P<0.05) differences in the effects produced by both doses were only observed at 60- and 240-minutes intervals.

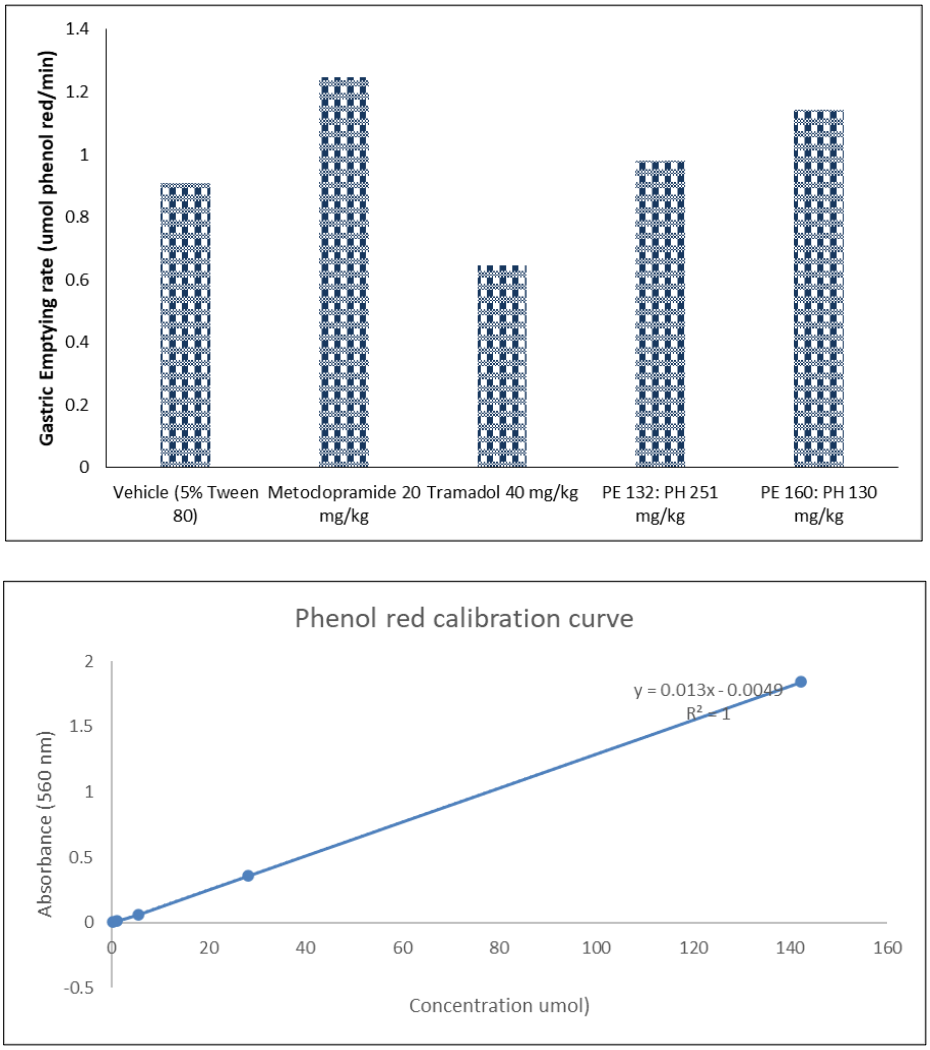


Figure 4 Gastric emptying rate of phytoestrogen rich fraction

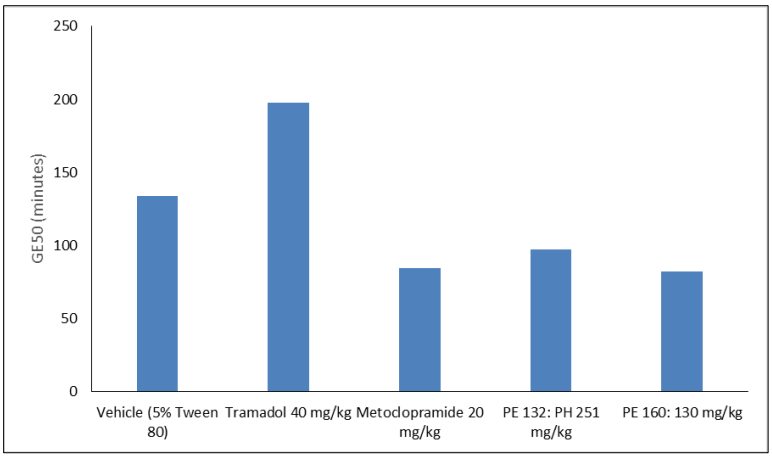


Figure 5 Time required for 50 percent gastric emptying

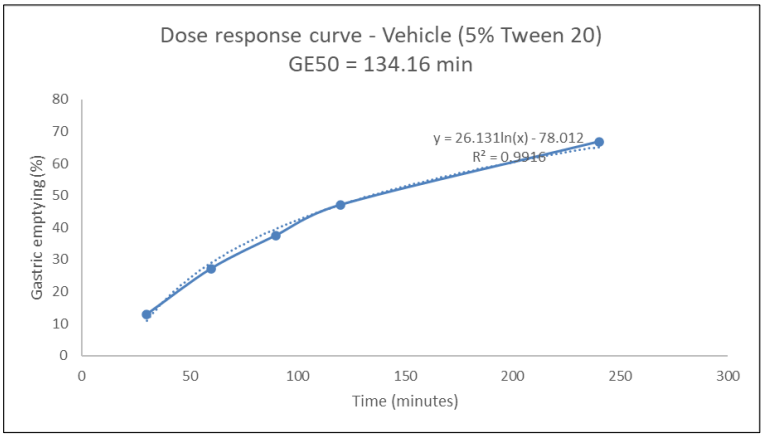


Figure 5a Time required for 50 percent gastric emptying for vehicle control

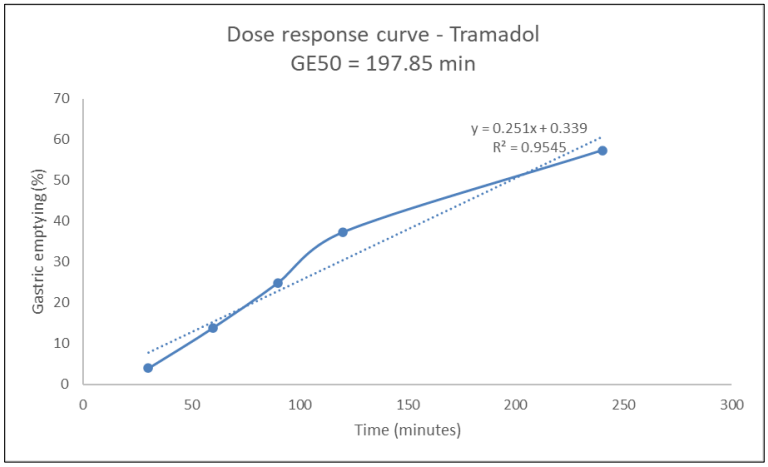


Figure 5b Time required for 50 percent gastric emptying for Tramadol

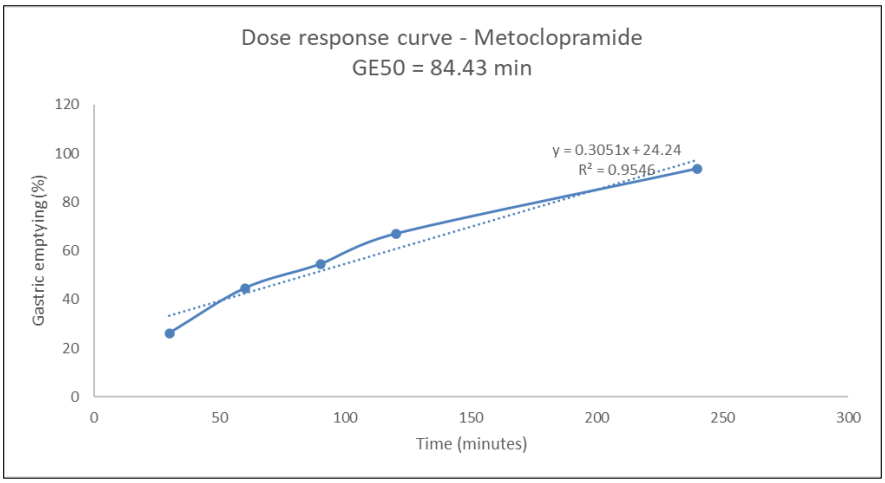


Figure 5c Time required for 50 percent gastric emptying for metoclopramide

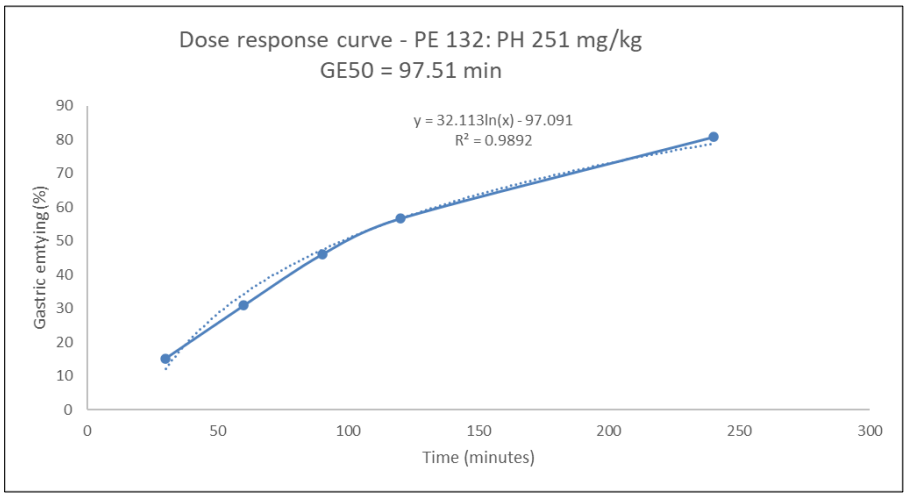


Figure 5d Time required for 50 percent gastric emptying for PE 132: PH 251 mg/kg phytoestrogen: phenol rich fractions combination

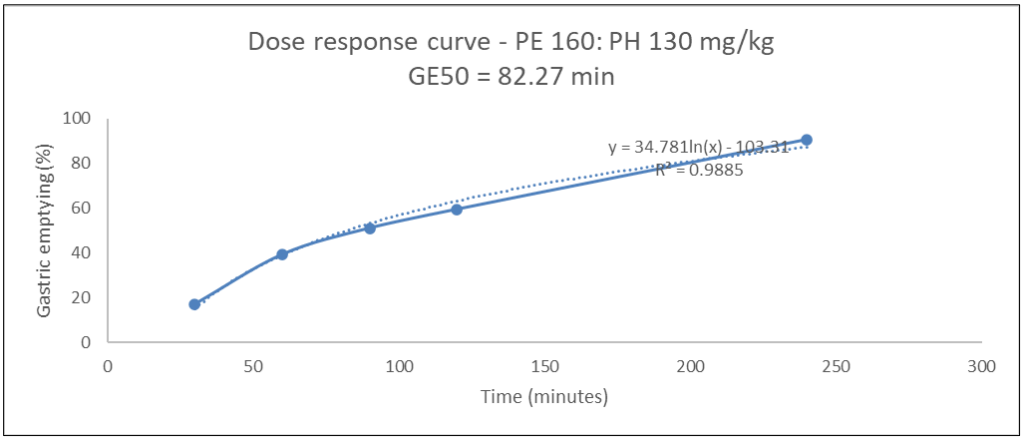


Figure 5e Time required for 50 percent gastric emptying for PE 160:PH130 mg/kg phytoestrogen: phenol rich fractions combination

5. Discussion

Proper gastric emptying relies on synchronized activity of the upper stomach fundus, lower body, antrum, and pylorus. Gastric motility is governed by a complex network of neuronal and hormonal signaling. Various cell types, such as extrinsic innervation of the stomach, enteric nerves, glia, smooth muscle cells, interstitial cells of Cajal (ICC), and immune cells, are essential for normal gastric motility. Gastroparesis is a chronic disorder characterized by delayed gastric emptying of solids and liquids, without obstruction. The most prevalent types are idiopathic, diabetic, and postsurgical complications [18,19,20]. Most idiopathic patients are either obese or overweight [19]. A retrospective study indicated that weight gain during pregnancy and menopause significantly increased the likelihood of being diagnosed with diabetes and being overweight, which can predispose post-menopausal women to gastroparesis. Similarly, postmenopausal women undergoing sex hormone therapy experienced delayed gastric emptying compared with age-matched men. Delayed gastric emptying and reduced gastrointestinal motility can lead to functional gastrointestinal disorders such as indigestion and constipation. The role of nitric oxide in stomach motility is well established [6]. nNOS acts as an inhibitory neurotransmitter, and is crucial for stomach emptying. Animal studies have shown that nNOS dimerization is vital for gastric motility, and alterations in nNOS function and expression may result in delayed gastric emptying in conditions such as diabetes, hyperlipidemia, oxidative stress, and, notably, chronic estrogen deficiency [6,2]. Evidence suggests that estrogens enhance both eNOS and nNOS functions, maintaining cardiovascular and neuronal functions, indicating that the function of nNOS in the stomach may be regulated by endogenous hormones [6,21]. Supporting this hypothesis, Shah et al. [11] found that estrogen supplementation improved nNOS expression, nNOS neurons, and ultimately nNOS function in adult female rats. The inhibitory effects of estrogen on gastric emptying are in contrast with the results obtained from the PERF of *Ochna schweinfurthiana*. This observation implies that the stimulatory effect of PERF is not mediated by the classical estrogen receptor signaling pathway. Other potential mechanisms explaining the prokinetic effect of PERF could involve smooth muscle contractions through increased cytosolic calcium, either by stimulating calcium influx or releasing calcium from intracellular stores, or both. The stimulating action might also result from other mechanisms, such as the increased sensitivity of the contractile apparatus to the existing concentration of calcium in contractile proteins.

6. Conclusion

The phenol and phytoestrogen-rich fraction of *Ochna schweinfurthiana* demonstrated a prokinetic effect on gastric emptying. This effect might be facilitated by various targets that regulate gastric motility. Additional research is necessary to determine the precise mechanism by which these phytocompounds enhance gastric emptying.

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.

Authors' contribution

UHO and DLA conceptualized the study. All authors performed the experiments. DLA, UHO and PC performed the analysis. All the authors drafted and approved the final version of the manuscript.

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