

Maternal toxicity and post-implantation assessments in rats gestationally exposed to *Polyscias fruticosa* leaf extract

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ABSTRACT

Background: *Polyscias fruticosa* (L.) Harms is used as a folk medicine across Afro-Asian regions of the world. Safety of *P. fruticosa* use in pregnancy remains completely unknown despite its extensive usage.

Objective: The study assessed maternal and post-implantation loss in pregnant rats gestationally exposed to *P. fruticosa* leaf extract (PFE).

Methods: Healthy female Wistar rats were co-habited (female:male; 6:2) with healthy fertile males. Confirmed pregnant rats were randomly re-assigned to normal saline (control, 5 ml/kg *po*), folic acid (5 mg/kg *po*), and PFE (100, 200, and 500 mg/kg *po*) and treated once daily for 15 gestational days. Maternal toxicity was assessed by cage-side observations, feed consumption, weight loss, relative organ/bodyweight ratio, biochemical assessment of liver and kidney function, morbidity, and death. Embryotoxicity was assessed by gross embryo assessment, embryo resorption, and brain/spinal cord histology.

Results: Two dams died from PFE (500 mg/kg) group. Feeding decreased across all groups as gestation progressed. No weight loss across all groups. Aspartate transaminase increased in PFE (200 and 500 mg/kg) relative to control. Alkaline phosphatase decreased ($P \leq 0.05$) in PFE compared to control. Direct bilirubin increased in PFE groups relative to control. Urea decreased in PFE groups relative to control. Creatinine levels decreased in PFE (100 mg/kg) relative to control but increased in PFE (500 mg) compared to control. Post-implantation loss increased in PFE (500 mg/kg) relative to control.

Conclusion: Gestational exposure to PFE (>100 mg/kg) produced risk of post-implantation loss and renal injury in dams; therefore, PFE should be avoided in pregnancy.

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Introduction

Use of herbal products, especially herbal medicines has increased immensely [1–3]. In poorer tropical regions of the world, such as in some parts of Africa and Asia, herbal medicine continues to be the main source of primary health care for most populations [4–6], while in the developed world it is used as a complement to conventional medical care. Many reports show that sensitive populations such as

pregnant mothers use herbal products in one way or the other to manage their pregnancies [7–9] with most of them having the belief that herbal products are safe alternatives of the conventional medical care [10]. For example, a survey revealed that well over 52% of expectant mothers used herbal products as a complement to other health care to manage their pregnancies [7,8,11]. In a study involving 374 herbal medicine practitioners, 74.3% were of

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the view that herbal medicines do not have adverse effects because it is organic, while 24.1% admitted that herbal medicines may produce adverse effects and that some of their clients reported adverse outcomes [10]. It is in response to these health threats from the use of herbal products that the World Health Organization has strongly recommended inclusion of herbal medicines in existing national and regional pharmacovigilance systems [10]. Further experts in the herbal product industry have advised users of herbal products to exercise maximum caution [12,13], since improper use of herbal products just like any other drug could pose serious health risk. Despite all these efforts to regulate and reduce herb-related adverse events, evidence still shows that hand-in-hand increased use of herbal products is accompanied with high incidence of herb-related adverse events [14,15], especially among sensitive groups. For example, use of some herbal products has been identified with reproductive toxicity [16–18] and embryo toxicity [19]. It was reported that a number of herbs used as part of Chinese herbal medicine to treat threatened abortions had no safety data with respect to maternal, reproductive, and developmental toxicity [13,20]. Hitherto herbs thought to be safe are now identified with developmental toxicity in rats [14,21]. A recent report showed that two-thirds of emergency cases in the US were attributed to the use of herbal products [22].

Many factors underlie the problem of herb-related adverse events in addition to the general belief that it is organic in nature and therefore safe [10]. Other factors include the high potential of herbal products to be self-medicated [5,8], especially in some African countries, where traditional and cultural beliefs strongly influence its use [4]; the seeming lack of research data on herbal products to inform proper regulation and lack of safety data on most commonly used herbal products [7,23].

Scientific evaluation of herbal medicines in the light of their ethnobotanical context of use in Africa and in Ghana in particular has improved substantially. Nonetheless, these improvements have overly focused on efficacy assessments to the neglect of safety. One of such important herbal plants with poor safety assessment is *Polyscias fruticosa* (L.) Harms (Syn. *Panax fruticosa* L., *Nothopanax fruticosa* Miq.; family: Araliaceae). *Polyscias fruticosa* is put to many ethnobotanical uses across Afro-Asian regions of the world. In Ghana, *Polyscias fruticosa* leaf preparation is drunk to treat inflammatory and upper respiratory disorders among all class

of people. To scientifically confirm or otherwise, it was shown that ethanol leaf extract of *P. fruticosa* exhibited anti-inflammatory, muco-suppressant, anti-tussive, and anti-asthma properties [24,25]. Aside Ghana, various crude preparations of *P. fruticosa* are used in Asia as tonic, anti-inflammatory, anti-toxin, anti-bacteria as well as a spice and a digestion agent [26]. As an example in Vietnam, apart from been eaten as salad, it is also used as a tonic to treat inflammation, ischemia, and to improve brain vasculature [27]. Also, a root extract of *P. fruticosa* is documented as a diuretic, febrifuge, treatment for dysentery, neuralgia, and rheumatic aches [28]. Ecologically, *Polyscias fruticosa* (L.) is widely distributed across Eastern Asia, Tropical islands of the Pacific Region [26] as well as in some African countries including Ghana [24,25]. Phytochemical fingerprints of *P. fruticosa* have been elaborated. Notably two oleanoic acid, saponins and polyacetylenes, were respectively isolated from leaves [29] and roots of *P. fruticosa* [26]. Also, three bisdesmosidic saponins were isolated from leaves of *P. fruticosa*, of which the first saponins was shown to exert inhibitory effect on α -amylase and α -glucosidase activity, and was thus speculated to be useful for diabetes treatment [27].

Although many studies have evaluated the pharmacological properties of *Polyscias fruticosa* in the light of various ethnobotanical context of use [24,25,27], these studies overly focused on efficacy of *P. fruticosa* but not its safety, especially in reproduction. It still remains to be established the safety of *P. fruticosa* with respect to maternal and embryo-fetal toxicity. This apparent research gap came to light upon a thorough PubMed and Google Scholar searches. To bridge this research gap and to provide rationale for follow-up studies on *P. fruticosa* as a potential herbal drug, this study investigated maternal and embryo-fetal toxicity in rats after gestational exposure of rats to crude *P. fruticosa* leaf extract (PFE).

Materials and Methods

Collection, identification, and authentication of medicinal plant

Leaves of *Polyscias fruticosa* were collected from the botanical gardens of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Leaves were identified and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/

HM/13/W010) [25,30] was deposited at KNUST herbarium for future reference.

Preparation of *Polyscias fruticosa* leaf extract

Fresh *Polyscias fruticosa* leaves (2.5639 kg) were washed with clean water, shade-dried completely, and pulverised into powder (1.8444 kg). The powder was soaked in absolute ethanol (4.8 L) in a volumetric flask for 72 hours, and then filtered. Ethanol was retrieved from the dark-green filtrate in a rotary evaporator (B'U'CHI Olibath B-485) at 50°C. The residue was dried completely in a desiccator containing activated silica gel. The crude extract yielded 62.979 g, given a percentage yield of 3.414%. The final crude extract obtained, was named PFE and was referred to as PFE throughout the study. Subsequently, PFE was fractionated into ethyl acetate, petroleum ether, and ethanol fractions. Thin layer chromatography (TLC) was conducted on each fraction to determine component spots and retardation factor by following a previous method [31] with some modifications. The ethanol fraction was used in all experiments. Subsequently, the ethanol fraction was analysed by using a gas chromatography–mass spectrometry (GC–MS) technique under the following operating conditions: [Injector-1177 (splitless mode); Injector temperature-250°C; Injection volume-2 µl; Carrier Gas--Helium at Constant flow of 1.0 ml/minute; Analytical Column--Hewlett Packard-5 30 m, 0.25 mm, 0.25 µm; MS Acquisition range 30–650 m/z; Ionization mode–electronic ionisation; and column oven temperature (250°C), rate (10°C/minute), hold (7), and total (30)].

Acquisition of experimental animals and husbandry

Healthy 8 weeks old, female (150–180 g) and male (120–200 g) Wistar rats were purchased from the animal breeding unit of Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. To afford adequate acclimatization, experimental animals were kept at the animal house of the School of Biological Sciences, University of Cape Coast for 2 weeks before the start of experiments. All experimental animals were maintained under ambient conditions of temperature, relative humidity, and 12 hours light/dark cycle, and housed in sanitized aluminium cages (20 × 15 × 8 cm) with base dressing of sawdust as bedding. They were given access to standard pellet diet (Grower Mash, Essaar, Ghana) and water *ad libitum*; however, these conditions were varied where necessary to meet specific experimental requirements. Experimental

animals were handled in strict compliance with the guide for the Care and Humane Use of Laboratory Animals (National Research Council, 1996) as well as specific institutional and national guidelines on the humane use and handling of experimental animals in scientific experimentation.

Co-habitation and confirmation of pregnancy

Healthy dams without confirmed stage of oestrous cycle were co-habited with confirmed fertile male rats at a female:male ratio of 6:2. Post-coitus confirmation of pregnancy was done by detection of a vaginal copulatory plug as previously described [4,32]. All confirmed pregnant dams were randomly re-assigned to one of the five treatment groups. The day of detection of vaginal copulatory plug was considered day 1 of pregnancy.

Group allocation and bodyweight measurements

Experimental animals were randomly assigned to one of the five groups {control [normal saline (NS), 5 ml/kg], folic acid (5 mg/kg), PFE (100, 200, and 500 mg/kg, respectively)}. Each group had four dams except PFE (200 mg/kg) which had five dams. Bodyweight of dams were measured before treatment started and also at gestational days (GDs) 5, 10, and 15. Dams in each group were uniquely labelled.

Experimental design and dose administration

Dams in each group were dosed by oral gavage. Dosing was done once every morning at 8.30 am from GD 1–14 of gestation. Dams in the respective groups were treated as shown below:

Control group (5 ml/kg of NS *po*) once daily + free access to food and water

PFE (100 mg/kg *po*) once daily + free access to food and water

PFE (200 mg/kg *po*) once daily + free access to food and water

PFE (500 mg/kg *po*) once daily + free access to food and water

Folic acid (5 mg/kg *po*) once daily + free access to food and water

Measurement of food consumption

Feed for dams in each group was quantified before, and then re-quantified 24 hours later to determine average food consumption per day/group. Average

Table 1. TLC analyses of PFE.

Ethanol extract					
Solvent system	Ratio	Resolution	Number of spots	Rf	*Spots colour
Ethanol:Ethyl acetate	1:5	Good	4	0.95	Light green
				0.73	Light yellow
				0.32	Light brown
				0.12	Light brown
Solvent distance = 7.7 cm					
Ethanol:Ethyl acetate	1:3	Not well resolved	—	—	—
Ethanol:Ethyl acetate	1:1	Not well resolved	—	—	—

*Rf = retardation factor. Under ultra violet light (254 nm).

food consumption for each group at 5, 10, and 15 GDs was determined and compared statistically.

Cage-side observations

Dams in each group were inspected twice (morning and afternoon) daily for changes in skin, fur, eyes, mucous membrane, piloerection, aversion to handling and breathing as previously described [4,33]. Only behavioural and signs that occurred consistently in the morning and afternoon were considered to be related to treatment; and therefore, could be of toxicological significance.

Determination of mortality and morbidity among dams

Dead and moribund dams were determined for each treatment group at the end of treatment. Morbidity was defined to mean any animal that displayed a spectrum of physical or behavioural signs that impaired mobility, feeding, and response to touch.

Biochemical analysis

After anaesthesia followed by surgical opening of each dam as previously described [33], blood samples were collected from the inferior vena cava, processed, and analysed for liver and kidney function determinants as previously described [33].

Determination of relative organ weights

Heart, liver, and kidney (both right and left) were isolated from each dam, weighed, and inspected for any gross lesions of toxicological relevance. Organ to bodyweight ratios were determined for dams in each group and compared.

Post-implantation assessment

At GD 15, dams were euthanized by intravenous injection of sodium pentobarbital (40 mg/kg),

caesarean-sectioned, gravid uterus isolated, weighed, surgically opened, and contents examined as previously reported [33]. Total implantation sites, number of embryos, and number of resorptions were determined for animals in each group. Post-implantation loss (the difference between total implantation sites and number of embryos) and % post-implantation loss (number of resorptions/total implantation sites × 100) were determined for each group. Embryos were removed, counted, and weighed. Embryos were closely inspected for external abnormalities as previously described [34,35].

Histological assessment of embryo-fetal brain and spinal cord

At GD 15, dams in each group were weighed, anaesthetised, sacrificed, and caesarean-sectioned. Gravid uteri were isolated and weighed. Foetuses were harvested, inspected grossly, and fixed in Bouin's solution followed by physical examinations as previously reported [33]. Representative brain and spinal cord samples from each group were collected fixed, sectioned, followed by hematoxylin and eosin staining as previously described [36]. Three independent histo-pathologists microscopically studied the histological sections and final description of histological sections was by consensus.

Statistical analysis

Statistical analysis on data was done by using Graph Pad Prism Version 6 for Windows (Graph Pad Software, San Diego, CA). Some of the data were presented in tables as mean ± standard deviation (SD). Mean comparison between groups was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. $P \leq 0.05$ was considered statistically significant in all analyses.

Table 2. GC–MS analysis of PFE showing target compounds.

Peak name	RT	Quan ions	Area	Amount/Rf
Trans-, alpha-, -Bergamoten	14,793	119.0	5,967	5,967 counts
1 H-3a, 7-Methanoazulene	16,039	107.1	3,598	3,598 counts
Hematoporphyrin ix	16,313	202.9	1,189	1,189 counts
3,7,11,15-Tetramethyl-2-	18,638	67.0	7,960	7,960 counts
5-Nonadeeen-1-ol	18,891	67.0	1,619	1,619 counts
3,7,11,15-Tetramethyl-2-	19,079	67.0	2,306	2,306 counts
Falcarinol	20,625	91.2	4,6378	46,378 counts
3,7,11,15-Tetramethyl-2-	21,340	41.2	8,305	8,305 counts

Rf = retardation factor; RT = retention time.

Results

Phytochemical analyzes of PFE

Saponins, alkaloids, cyanogenic glycosides, and sterols were identified in PFE. TLC analysis showed four spots (Table 1). GC–MS profiling of PFE showed 12 peaks out of which 8 were identical to library compounds (Table 2).

Effect of treatments on maternal food consumption

Across all treatment groups, food consumption declined from GD 1 to 15. Although there were differences in food consumption among the groups; however, the differences were not statistically significant ($P \geq 0.05$) (Fig. 1).

Effect of treatments on maternal bodyweight gain

Although PFE (200 mg/kg)-treated dams had a higher net maternal bodyweight gain compared to control dams (Fig. 2); however, the mean difference was not statistically significant ($P \geq 0.05$).

PFE (100 and 500 mg/kg) had lower maternal net bodyweight gain compared to control dams but the mean difference was not statistically significant ($P \geq 0.05$). Comparatively, folic-acid treated dams had the highest net maternal bodyweight gain compared to control and PFE groups; and the mean difference was statistically significant ($P \leq 0.05$) (Fig. 2). Also, there were no significant ($P \geq 0.05$) differences between mean organ (liver, heart, and kidney) weights at GD 15 between control dams and treatment groups (PFE and folic acid). Across all groups, right kidney weights were higher than left kidney weights. Although there were differences between the relative organ weights of control and treatment groups (PFE and folic acid), these differences were not statistically significant ($P \geq 0.05$) (Table 3).

Effect of treatments on behaviour and physical activity of dams

There were no treatment-related changes in physical activity among control dams and treatment

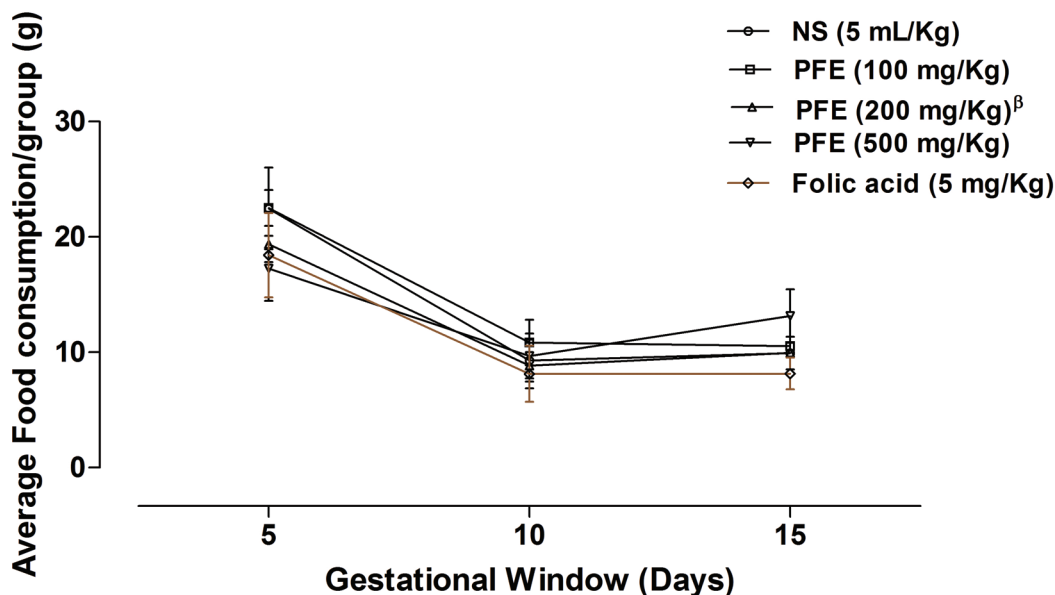


Figure 1. Effect of treatments on food consumption at GDs 5, 10, and 15. Each point is the mean \pm SD of the amount of feed (g) consumed at each gestational window; $n = 4$; $^{\beta}n = 5$.

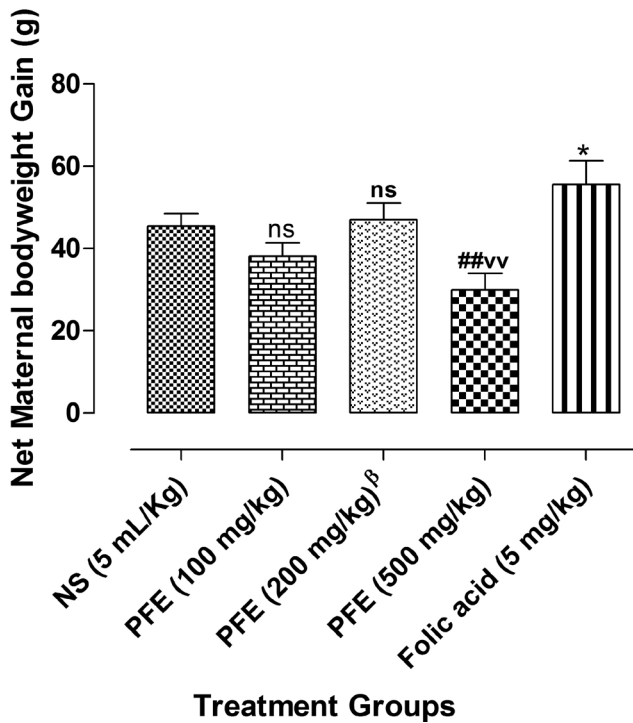


Figure 2. Effect of treatments on net bodyweight gain at GD 15; each bar is the mean \pm SD weight gain by dams in each treatment group. $N = 4$; $^{\beta}n = 5$. $P \leq 0.05$ was considered statistically significant in all analysis. $^{vv}P \leq 0.01$ (Folic acid vs. PFE); $^{##}P \leq 0.01$ (NS vs. PFE); $^{*}P \leq 0.05$ (NS vs. Folic acid); ns = not statistically significant ($P > 0.05$).

groups (PFE 100 and 200, and folic acid) except in PFE (500 mg/kg), where two dams were found to be weak, non-responsive to physical touch. No piloerection was observed in all groups except the two moribund dams in PFE (500 mg/kg) group. At GD 10, the two moribund dams in the PFE (500 mg/kg) group died leading to a 50% mortality compared to 100% survival rate for control, PFE (100 and 200 mg/kg), and folic acid groups over the same treatment period (Table 4). Autopsy report on the two dead dams from PFE (500 mg/kg) treatment group by two independent pathologists did not implicate PFE treatment but attributed their deaths to mishandling during drug administration.

Effect of PFE treatment on liver function

Alanine transaminase (ALT) was comparable across all treatments. Aspartate transaminase (AST) significantly ($P \leq 0.05$) decreased in low dose PFE (100 mg/kg) group relative to control, but marginally increased in median and high dose PFE (200 and 500 mg/kg) groups relative to control and folic acid groups (Table 5). Total protein (TP) and globin (GLO) marginally increased in low-and median-dose PFE (100 and 200 mg/kg) groups relative to control and folic acid groups but decreased in high-dose PFE (500 mg/kg) relative to control. Albumin (ALB) marginally increased in PFE (100–500 mg/kg) groups relative to control. Both total

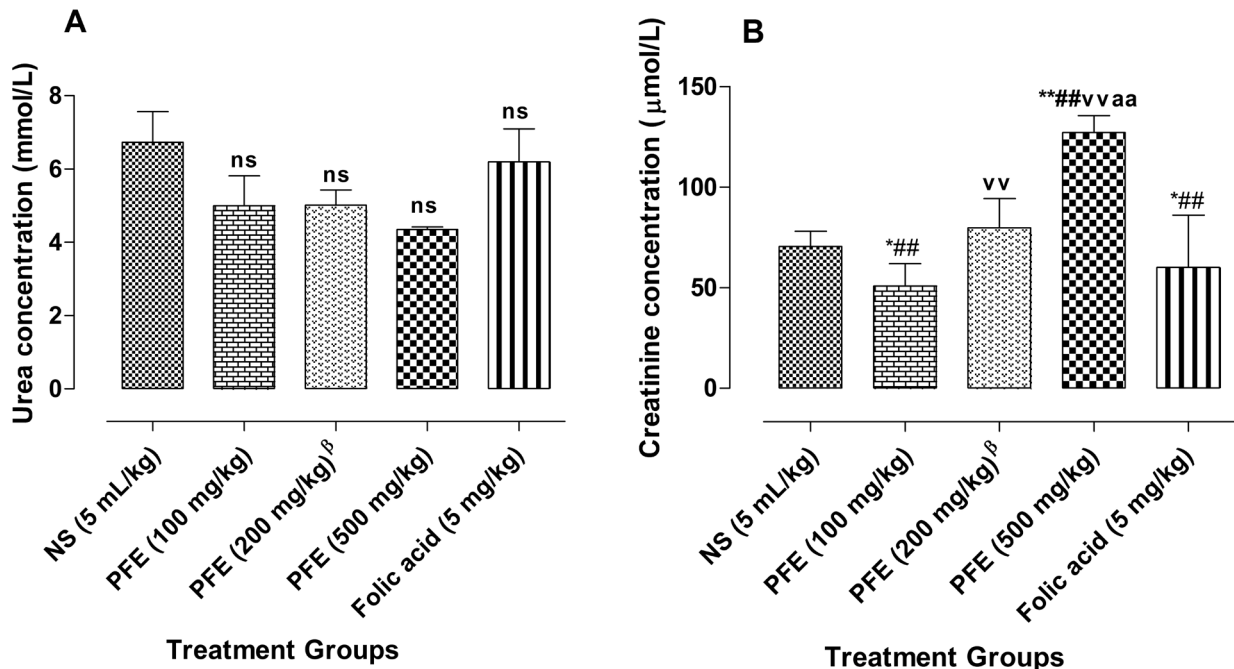


Figure 3. The effect of treatments on serum urea (A) and creatinine (B) levels determined at GD 15. Each bar is the mean \pm SD. $n = 4$; $^{\beta}n = 5$. Values are Mean \pm SD. $n = 4$; $^{\beta}n = 5$. $P \leq 0.05$ was considered statistically significant for all statistical analyses. ns = not significant; $^{*}P \leq 0.05$ (NS vs. PFE); $^{##}P \leq 0.01$ (NS vs. PFE); $^{##}P \leq 0.01$ (PFE vs. Folic acid); $^{vv}P \leq 0.01$ PFE (100 vs. 200 mg); $^{aa}P \leq 0.01$ PFE (200 vs. 500 mg).

Table 3. Effect of treatments on organ/bodyweight ratio at GD 15.

Treatment groups	Maternal bodyweight at GD 15 (g)	*Relative organ weight × 10 ⁻² (%)			
		Liver	Heart	Right kidney	Left kidney
NS (5 ml/kg)	213.77 ± 18.114	3.71 ± 0.335	0.32 ± 0.017	0.33 ± 0.031	0.30 ± 0.026
PFE (mg/kg)					
100	194.99 ± 5.817 [#]	3.89 ± 0.686 ^{ns}	0.36 ± 0.121 ^{ns}	0.33 ± 0.071 ^{ns}	0.30 ± 0.074 ^{ns}
200 ^β	190.29 ± 20.998 ^{##}	4.17 ± 0.830 ^{ns}	0.36 ± 0.055 ^{ns}	0.37 ± 0.058 ^{ns}	0.35 ± 0.055 ^{ns}
500	207.44 ± 6.463 ^{ns}	3.80 ± 0.049 ^{ns}	0.31 ± 0.007 ^{ns}	0.32 ± 0.042 ^{ns}	0.30 ± 0.028 ^{ns}
Folic acid (5 mg/kg)	194.20 ± 30.345 [*]	3.89 ± 0.347 ^{ns}	0.31 ± 0.081 ^{ns}	0.32 ± 0.044 ^{ns}	0.28 ± 0.033 ^{ns}

Each value is the mean ± SD. N = 4; ^βn = 5. one-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze mean differences between groups. P ≤ 0.05 was considered statistically significant for all comparisons. ns = not statistically significant (P > 0.05). *maternal bodyweight at GD 15/respective mean organ weight (liver, heart, right, and left kidneys)/group × 100.

and direct bilirubin (TB and DB) marginally increased in low-and median-dose PFE (100 and 200 mg/kg) and folic acid groups relative to control, but significantly (P ≤ 0.05) increased in PFE (500 mg/kg) group compared to control. Indirect bilirubin (IB) marginally increased in PFE (100–500 mg/kg) and folic acid groups compared to control. Alkaline phosphatase (ALP) significantly (P ≤ 0.05) decreased in PFE (100–500 mg/kg) groups relative to control and folic acid groups. Gamma-glutamyltransferase (GGT) marginally decreased in low-dose PFE (100 mg/kg) but increased marginally in PFE (200 and 500 mg/kg) and folic acid groups compared to control (Table 5).

Effect of PFE treatment on kidney function

Urea concentration decreased in PFE (100, 200, and 500 mg/kg) especially in PFE (500 mg/kg) compared to control and folic acid groups; however, the mean difference was statistically insignificant (P ≥ 0.05) (Fig. 3A). Folic acid and PFE (100 mg/kg) decreased mean creatinine concentration compared to control, though the mean difference was not significant statistically (P ≥ 0.05). PFE (200 and 500 mg/kg) treatments increased significantly (P ≤ 0.05) mean creatinine concentration compared to control-and folic acid-treated dams (Fig. 3B).

Effect of PFE treatment on post-implantation development

Mean gravid uterine weight was not significantly (P ≥ 0.05) different across treatment groups (PFE and folic

acid) and control. There was post-implantation loss in high-dose PFE (500 mg/kg) relative to control, folic acid, and low-and median-dose PFE (100 and 200 mg/kg). Except high-dose PFE (500 mg/kg) group, all the remaining treatment groups (low- and median-PFE, 100 and 200 mg/kg and folic acid) together with control group had 0% post-implantation loss (Table 6). A gross physical examination of embryos across all groups showed no observable anatomical malformations or deformations that could be related to treatment.

Histological assessment of brain and spinal cords of embryos after “in utero” exposure to treatments

Histological examination of brain and spinal cord sections of representative embryos showed no histo-morphological alterations of serious toxicological relevance except extensive eosinophilic cytoplasm and basophilic nuclei (Fig. 4).

Discussion

This study assessed the safety of PFE in pregnancy; specifically maternal toxicity and embryo-fetal toxicity post—“in utero exposure” was investigated. Hand-in-hand use of herbal medicine and herb-related adverse events have increased worldwide [4]. Crude preparations of *P. fruticosa* is used as a herbal medicine by all class of people including special populations (pregnant mothers, children, and the elderly) in Afro-Asian countries to treat many diseases including upper respiratory disorders [25,30] mostly by ingestion of deco-

Table 4. Effect of treatments on liver indices of dams at GD 15

Treatment groups	Number of pregnant rats	Deaths during treatment	Number of survived pregnant rats	Live index (%)
NS (5 ml/kg)	4	0	4	100.00
PFE (mg/kg)				
100	4	0	4	100.00
200*	5	0	5	100.00
500	4	2	2	50.00
Folic acid (5 mg/kg)	4	0	4	100.00

*n = 5.

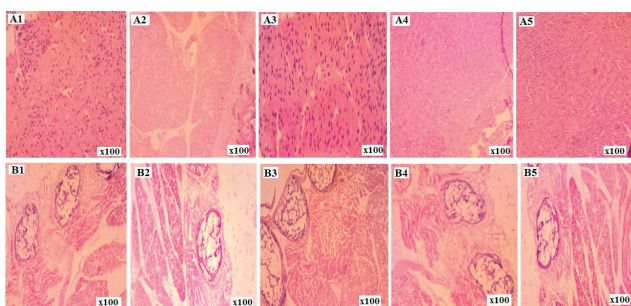


Figure 4. Each histomicrograph is a representative section of embryo-fetal brain (A1–A5) and spinal cord (B1–B5) after dams were gestationally exposed to treatments for 15 days and fetuses removed and examined histologically. Generally sections showed normal eosinophilic cytoplasm with basophilic nuclei and normal vascularization with normal nuclei within the soma of the neurons surrounded by glial cells and developing neuroglia. However, there was marked cellular disorganization and distortion of cortical architecture with interspersions of neuronal and glial cells layers in A2–A4 (PFE treatments) compared to A1 (normal control) and A5 (folic acid). Also, focal areas in A3 and A4 showed remarkable vacuolation compared to A1 and A5. (B) Histomicrographs of representative sections of fetal vertebral column showing unremarkable histomorphology with moderate mineralization (X) in B2, B3, and B4 compared to B1 and B5. A1 and B1 = normal saline (5 ml/kg *po*), A2 and B2 = PFE (100 mg/kg *po*), A3 and B3 = PFE (200 mg/kg *po*), A4 and B4 = PFE (500 mg/kg *po*), and A5 and B5 = folic acid (5 mg/kg *po*).

tions. Although scientific evidence abound to support the folk use of *P. fruticosa* for the treatment of many common diseases [24,37,38], no study assessed its safety in pregnancy. Previously acute and sub-acute toxicity studies on crude preparations of *P. fruticosa* conducted on non-pregnant rodents showed that it was safe at 2 g/kg [24], however, the present study showed

that gestational exposure of dams to PFE at doses >100 mg/kg may increase the risk of post-implantation loss and renal injury in pregnant rats. Maternal toxicity encompasses changes in motor activity, bodyweight loss, decline in feed and water consumption, changes in external body structures, e.g., hair, mucous membrane, moribundity, and finally death upon gestational exposure to a known drug or chemical entity [39–42]. In this study, although feed consumption decreased across all groups (treatment and control dams) and then stabilized between GD 10 and 15 as pregnancy advanced, this could not be related to treatment in view of the physiological demands of pregnancy [43] and the fact that decrease in food consumption failed to reflect in bodyweight loss as is often the case [44]. Dams in all groups gained weight and the differences in weight gain among the groups were not statistically significant compared to that of control dams except in the case of high-dose PFE (500 mg/kg) group, but as far as there was a gain in weight this could not be related to maternal toxicity as reported elsewhere [44]. Also, there were comparable relative organ weights across all treatment groups relative to control indicating that treatments did not produce organ hypertrophy and edema. This observation complements an earlier safety study on *P. fruticosa* in non-pregnant animals, where body and organ weights and other safety indices did not change across treatment groups compared to controls [25,30], suggesting that *P. fruticosa* is safe at least with respect to body and organ weight changes but what may happen at the cellular and tissue levels remained veiled.

To investigate any possible tissue/organ injury in relation to treatment, liver and kidney enzyme activities were monitored. Sudden increase in some tissue-specific enzymes is indicative of direct cell/tissue injury [45]. In this study, although PFE treat-

Table 5. Effect of treatments on liver enzyme activity at GD 15.

Parameter	NS (5 ml/kg)	PFE (mg/kg)			Folic acid (5 mg/kg)
		100	200 ^b	500	
ALT (μ/l)	123.75 ± 22.29	112.75 ± 8.66 ^{ns}	123.40 ± 7.02 ^{ns}	123.00 ± 2.83 ^{ns}	112.75 ± 5.56 ^{ns}
AST (μ/l)	208.00 ± 53.93	165.25 ± 22.77 ^{##}	215.60 ± 20.62 ^{vvaa}	236.50 ± 4.95 ^{vvaa}	145.75 ± 20.66 ^{***}
TP (g/l)	67.00 ± 8.87	72.25 ± 3.95 ^{ns}	71.40 ± 4.83 ^{ns}	62.00 ± 1.41 ^{ns}	59.25 ± 5.56 ^{ns}
ALB (g/l)	33.50 ± 5.26	38.00 ± 2.58 ^{ns}	36.20 ± 1.48 ^{ns}	35.50 ± 3.54 ^{ns}	30.50 ± 2.65 ^{ns}
GLO (g/l)	33.50 ± 5.26	34.25 ± 2.06 ^{ns}	35.20 ± 3.56 ^{ns}	26.50 ± 2.12 ^{ns}	28.75 ± 3.59 ^{ns}
TB (μmol/l)	5.10 ± 0.63	6.05 ± 0.79 ^{ns}	6.76 ± 2.75 ^{ns}	13.65 ± 2.05 ^{**}	7.78 ± 1.55 ^{ns}
DB (μmol/l)	2.83 ± 0.21	3.63 ± 0.44 ^{ns}	4.56 ± 2.57 ^{ns}	9.85 ± 0.354 ^{**}	3.33 ± 1.59 ^{ns}
IB (μmol/l)	2.28 ± 0.75	2.43 ± 0.43 ^{ns}	2.20 ± 0.264 ^{ns}	3.80 ± 1.70 ^{ns}	4.45 ± 1.10 ^{ns}
ALP (μ/l)	180.50 ± 14.48	92.75 ± 12.92 ^{***vvv}	157.80 ± 17.56 ^{vvaa}	151.50 ± 13.44 ^{vvaa}	224.00 ± 45.48 ^{***}
GGT (μ/l)	0.47 ± 0.050	0.34 ± 0.077 ^{ns}	0.50 ± 0.032 ^{ns}	0.50 ± 0.099 ^{ns}	0.52 ± 0.083 ^{ns}

Values are Mean ± SD. *N* = 4; ^b*n* = 5. The level of significance was established using one-way ANOVA followed by Bonferroni's multiple comparison test. *P* ≤ 0.05 was considered significant for comparison of mean differences between treatments. ns = not statistically significant (*P* > 0.05). ^{##}*P* ≤ 0.01 (NS vs. PFE); ^{**}*P* ≤ 0.05 (NS vs. Folic acid); ^{vv}*P* ≤ 0.01 (Folic acid vs. PFE); ^{aa}*P* ≤ 0.01 [PFE (100 mg) vs. PFE (200 and 500 mg)].

Table 6. Developmental toxicity assessments after daily gestational drug exposure to dams for 15 days.

Parameter	NS (5 ml/kg)	PFE (mg/kg)			Folic acid (5 mg/kg)
		100	200 ^b	500	
Gravid uterine weight (g)	19.68 ± 0.622	15.90 ± 0.421	17.58 ± 0.723	15.63 ± 0.944	21.70 ± 0.859
Total implantation sites	8.25 ± 0.304	7.25 ± 0.957	6.20 ± 0.387	8.00 ± 0.000	7.75 ± 0.5
No. of embryos	8.25 ± 0.304	7.25 ± 0.957	6.20 ± 0.387	5.00 ± 0.243*	7.75 ± 1.5
^a Post-implantation loss	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	3.00 ± 0.243	0.00 ± 0.000
^b % post-implantation loss	0.00	0.00	0.00	37.5* [#]	0.00
Embryo weight (g)	9.87 ± 0.407	6.58 ± 0.201	8.76 ± 0.017	6.12 ± 0.243	10.86 ± 0.590
Average weight per embryo (g)	1.26 ± 0.224	0.90 ± 0.065	1.38 ± 0.230	1.35 ± 0.297	1.44 ± 0.174
Embryo weight to mother weight (%)	0.60 ± 0.008	0.47 ± 0.006	0.73 ± 0.021	0.65 ± 0.027	0.76 ± 0.165

Each value is the mean ± SD. *n* = 4; ^bmeans *n* = 5. The level of significance was established using one-way ANOVA followed by Bonferroni's multiple comparison test. ^aTotal implantation sites minus number of embryos; ^bNumber of resorptions/total implantation sites × 100; *P* ≤ 0.05 was considered statistically significant for comparison of mean difference between treatments. **P* ≤ 0.05 (NS vs. PFE); [#]*P* ≤ 0.05 (Folic acid vs. PFE).

ment marginally increased serum levels of some liver enzymes compared to control, the differences were not significant statistically; therefore, could not be inferred to indicate liver injury particularly in view of the fact that these liver enzymes are not liver-specific. However, PFE significantly decreased the serum levels of key liver enzymes conventionally used to assess liver function, including ALP and AST. Of note, increase in serum ALP is reported to be characteristic of bile duct obstruction, intrahepatic cholestasis, and hepatic damage [46]. Also, serum ALP levels at any point reflect combined activity of many ALP isoenzymes from many organs including the liver, bone, kidneys, placenta, and intestinal lining; therefore, PFE treatment-related decrease in ALP suggest that PFE have no adverse effects on the liver, placenta, and other organs during pregnancy in view of the multi-organ source of ALP and the fact that during pregnancy, the placenta may account for about half the total serum ALP [47]. AST partakes in energy production via its role in the Krebs's cycle. In cells, AST is located in the cytoplasmic and mitochondrial compartments. It is widely distributed in many cells including liver, heart, kidney, pancreas, skeletal muscles, as well as in red blood cells [48]. Cellular damage causes release of AST in serum. Serum AST level at any point is proportional to the degree of cellular damage and may fluctuate in sharp response to cellular damage and disease severity [49]. In the present study it was observed that, low-dose PFE (100 mg/kg) decreased serum AST compared to control suggesting that PFE at doses ≤100 mg/kg may be tolerated in pregnant animals without liver toxicity concerns. However, increasing PFE doses (200 and 500 mg/kg) marginally increased serum AST levels relative to control, perhaps indicating that PFE may

be hepatotoxic at doses ≥200 mg/kg in pregnant animals contrary to previous report in non-pregnant animals [30]. Urea undergoes renal excretion as the main means of eliminating nitrogen. Renal impairment causes blood urea concentration to rise exponentially, while creatinine accumulates in blood in sharp response to renal damage. Results from this study showed decreased urea concentration in PFE (100, 200, and 500 mg/kg) groups relative to control and folic-acid groups showing that with specific reference to urea PFE may not pose risk of renal toxicity in pregnancy. However, with respect to creatinine concentration, PFE exhibited a biphasic effect in that at low-dose PFE (100 mg/kg) it decreased creatinine concentration relative to control but at higher doses (200–500 mg/kg) increased creatinine concentration relative to control suggesting that safety of PFE with respect to kidney function in pregnancy is dose-related; and therefore, gestational exposure to PFE above 100 mg/kg should be avoided.

During pregnancy, maternal conditions affect the developing neonate since the neonate depends directly on the mother for nutrients and excretion of waste materials via the placenta. Folic acid is conventionally used during pregnancy in humans for many reasons including prevention of neural tube disorders [4,50], and normal maintenance of key physiological processes for proper development of the neonate [51]. In the present study, folic acid group was used as a positive control for assessment of PFE use during pregnancy. In this study, all the embryo-fetal indices did not show significant differences across treatments and control groups except in % post-implantation loss and brain/spinal cord histological assessments which showed that high PFE dose (≥500 mg/kg) may produce risk of implantation loss and embryotoxicity. Effects of

herbal drugs just as their conventional counterparts relate not only to their chemical nature but also their chemical composition. The present results showed that PFE contains cyanogenic glycosides, saponins, alkaloids, and sterols and these observations support earlier reports [25,30]. GC–MS analysis of PFE showed 12 peaks, out of which 8 matched library compounds. PFE contains cyanogenic glycosides, and it has been shown that cyanogenic glycosides may hydrolytically release endogenous hydrocyanic acid (HCN) at levels that may cause toxicity [52]. HCN is readily absorbed and distributed in systemic circulation. HCN competitively inhibits cytochrome C oxidase, a crucial enzyme in the electron transport chain, leading to blockade of oxygen-reducing cofactor of proteins [52]. HCN-dependent cessation decreases oxygen utilization and triggers compensatory mechanisms such as activation of inefficient alternative energy generation such as anaerobic respiration. As a result, there is energy depletion, accumulation of lactic acid, metabolic acidosis, and cell death. The potential toxicity of cyanogenic glycosides especially in economically important crops such as *Manihot esculenta* necessitated issuance of acute reference doses for cyanide in cyanogenic glycoside-containing products by the Joint Expert Committee on Food Additives. Another phytochemical component of PFE was saponins, which is generally shown to interact chemically with cholesterol to form complexes believed to be responsible for hemolysis [53]. Also, saponins interact with living cell membranes to produce immunologic effects [53]. From our results, we suggest that *P. fruticosa* should not be used during pregnancy at any dose because of the following reasons. Put together, we suggest that *P. fruticosa* should not be used in pregnancy despite the fact that it did not produce maternal and post-implantation loss at 100 mg/kg.

Conclusion

Gestational exposure of PFE to pregnant dams at doses above 100 mg/kg may produce renal injury and post-implantation loss in pregnant animals; therefore, *P. fruticosa* should be avoided in pregnancy.

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Conflict of Interest

The authors declare no conflict of interest.

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