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RESEARCH ARTICLE

In-utero exposure to ripe *Carica papaya* seed resulted in postnatal outcomes from only first and second trimesters in rats

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Abstract

Limited information is available on the effect of ripe *Carica papaya* seed on postnatal outcomes. The purpose of this study was to examine its effect on pregnancy outcome, postnatal growth, somatic landmarks, and anogenital index (AGI) in offspring of rats exposed to the extract during three trimesters as well as a pretreatment group. Pregnant rats were randomly distributed into five experimental groups: Control = administered olive oil (vehicle); Pretreatment = administered 50mg/kg of chloroform extract of *Carica papaya* seed (CECS)extract for 7days (before mating to complete at least an oestrus cycle); D7 = administered 50mg/kg of the extract from gestation day 1- 7 (first trimester); D14= administered 50mg/kg of the extract from GD 1-14 (second trimester); D21= administered 50mg/kg of the extract from GD 1-21 (third trimester). Pregnancy outcomes observed were length of gestation, litter size, litter birth weight, stillbirth incidences. The pre-implantation and post-implantation losses were also calculated. Offspring postnatal developments and somatic landmarks were also observed while the AGI was calculated. Data were analysed using, ANOVA and Student's t-test at p=0.05.

Treatment with CECS during pregnancy: increased post-implantation loss in the third trimester; increased litter birth weight and increased post-natal weights occurred in the pretreatment, first, and second trimesters; while delayed somatic landmark in age of eye opening occurred in offspring of first and second trimester treated rats and delayed age of fur appearance occurred in offspring from second trimester rats while an earlier onset of fur appearance occurred in offspring of first trimester exposed rats. Treatment did not affect the AGI on postnatal day 4 in the offspring exposed to the extract at pretreatment and first trimester of pregnancy. The achieved results indicate that caution should be taken when recommending the plant extract during pregnancy as abortions could occur if the extract is used throughout pregnancy even if they are from ripe fruits.

INTRODUCTION

List of abbreviations

CECS= chloroform extract of *Carica papaya* seed, AGI= anogenital index, GD= gestation day, PND= postnatal day

Papaya (*Carica papaya* L.) seed has been identified to have anti-fertility, anti-implantation as well as abortificient properties in female albino rats (Adebiyi et al., 2002, Oderinde et al., 2002, Raji et al., 2005). These scientific claims however have not affected the perception of people in Nigeria, towards its consumption either as food or as medication even in pregnancy. A study from the Northern part of Nigeria revealed that the fermented

Carica papaya seeds have been used to produce an indigenous Nigerian food condiment called 'daddawa', the Hausa word for a fermented food condiment akin to locust beans. The seeds are also used by the Yewa people of Ogun State, South-western Nigeria, in the treatment or prevention of yellow fever in new born babies (Faleyimu and Oluwalana, 2008). The Esan people of Edo State use it in the treatment of tuberculosis (Okoli et al., 2007). *Carica papaya* seed has an historical as well as extensive use in the traditional management of diabetes and obesity and it is also prescribed by Yoruba herbalists in the South-Western part of Nigeria (Gill, 1992, Adeneye and Olagunju, 2009). Recent ethnobotanical survey, conducted has also revealed that hot infusion of *Carica papaya* seeds is used by Yoruba herbalists in the treatment of poison related liver and renal diseases and this has been scientifically proven (Olagunju et al.,2009). More recently, research revealed that the Nsukka indigenes of Eastern Nigeria chew the dry seed of *Carica papaya* to alleviate nagging headache (migraine) and in reducing swollen wounds and in reducing high blood pressure (Anaga and Onehi, 2010).

A papaya seed extract Caricarpyl-99 alkaloid extract is used in the Nigerian herbal medicine for the treatment of malaria, typhoid fever and other bacterial infections (Udoh et al., 2009). There are also claims of *Carica papaya* seed being used in anti-malaria drug preparations in Cameroun (Titanji et al., 2008). Chewing the seeds of ripe pawpaw fruit also helps to clear nasal congestion (Ayoola and Adeyeye, 2010) and its hematological properties have also been studied (Ikpeme et al., 2011).

Literature reviews suggest that the aqueous and chloroform extracts are non-toxic. However, the chloroform extract of *Carica papaya* seed has been more effective in male reproductive studies than the aqueous, methanol, ethanol, or ethyl acetate extractions especially in the quest for a contraceptive drug (Lohiya and Goyal, 1992, Lohiya et al., 1994a, Lohiya et al., 1994b, Chinoy et al., 1995, Lohiya et al., 1999, Pathak et al., 2000, Mishra et al., 2000, Sharma et al., 2000, Rajalakshmi and Sharma, 2001).

In a study by Raji et al., (2005), chloroform extract of *Carica papaya* seeds was administered to female rats as follows: Group 1; administration of extract was for two weeks before mating (pre-coital), Group 2; received extract from day 1 of pregnancy till term (post-coital) Group 3; received the extract for two weeks before mating and thereafter throughout term (pre- and post-coital). The study reported that the extract has anti-implantation and abortifacient properties in female albino rats. Despite the abortifacient property of the *Carica papaya* seed, it is still in use in folklore medicine during pregnancy. Our study is therefore aimed at looking into the effect of chloroform extract of *Carica papaya* seeds in each trimester of pregnancy as well as the gestational outcome and post-natal development in rats.

MATERIAL AND METHODS

Extraction of plant material. Seeds of *Carica papaya* were obtained from ripe fruits harvested on a farmland in Ibadan and identified and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen (FRIN 108041) was deposited. The seeds were air-dried and ground into powder. Chloroform extraction was done using soxhlet extractor at 58^0C for 12x 3. The filtrate obtained was concentrated under reduced pressure and yielded a dark brown semi-solid mass (w/w, 6%), which was used in this study.

Experimental animals. This study was conducted in accordance with the Use of Laboratory Animals committee of the Central Animal House, University of Ibadan in accordance with the NIH guidelines. 25 non-pregnant sexually mature female Wistar rats weighing between 190-220g were purchased from the Central Animal House, University of Ibadan, Nigeria.

Two consecutive regular four-day oestrus cycles were observed before the commencement of the study. Animals were maintained in an airy room under a controlled 12h:12h light/dark cycle. The rats were fed with standard rat chow (obtained from Ladokun feeds, Ibadan, Nigeria) and tap water ad libitum.

The oestrus cycles of the rats were monitored by examining daily vaginal smears. At prooestrus, female rats were caged with the male in the ratio of 2 females: 1male. In the morning between 7.00-9.00 a.m., vaginal smear was taken from each rat and observed under light microscope. Mating was deemed successful when spermatozoa were observed in the vaginal smear. The appearance of spermatozoa was taken as the first day of gestation (GD 1). On GD 20, pregnant females were transferred to bedding material.

An experimental pilot study was conducted to choose the best dose. 100mg/kg produced no litters thus a dose of 50mg/kg was chosen. This dose also had no lethal/ toxic effect on the female rats.

Experimental groups. Pregnant rats were randomly distributed into five experimental groups of five animals each: Control = administered olive oil (vehicle for the extract); Pretreatment = administered 50mg/kg of CECS for 7days (before mating to complete at least an oestrus cycle); D7 = administered 50mg/kg of CECS from GD 1-7 (first trimester); D14= administered 50mg/kg of CECS from GD 1-14 (second trimester); D21= administered 50mg/kg of CECS from GD 1-21 (third trimester).

Course of pregnancy. To evaluate for potential general toxicity, dams were daily monitored for mortality and signs of general toxicity. Weekly body weight and food consumption were determined during gestation and lactation with a calibrated electronic scale ((Ohaus scale corporation, U.S.A).

Pregnancy Outcome. Parturition was classified as difficult if delivery exceeded 2 hours and blood was present after delivery and dams failed to clean pups (Barrow, 1990). Pregnancy outcomes measured were length of gestation, litter size, litter birth weight, stillbirth incidence, and pre and post-implantation loses. The day of birth was considered postnatal day (PND) 1. The number of live offspring and the gender ratio were recorded. To keep the litters as homogenous as possible each dam nursed 9 male pups (Agnish and Keller, 1997, Chahoud and Paumgartten, 2009) throughout the lactation period to eliminate the effect of under-nutrition or over-nutrition of some of the pups, thus each group had 45 offspring each. After 21days, the pups were weaned to tap water and subsequently rat chow. After weaning, dams were killed by CO_2 inhalation and the number of uterine implantation sites was counted after staining the uteri with ammonium sulphide solution (10%, v/v) (Cavieres et al., 2002). The ovaries of rats were also dissected, and the number of corpora lutea counted. The two-horned uterus was removed and inspected for implantation sites.

The pre-implantation loss was calculated as follows:

number of corpora lutea - number of implantation sites

The post-implantation loss was calculated as follows:

number of implantation sites - number of fetuses

These values were calculated for each dam.

Determination of the corpora lutea was carried out using the method described by (Armanda-Dias et al., 2001).

For each litter, survival from implantation to the day after birth was calculated (Ulla et al., 2004).

Postnatal development. Pup viabilities were monitored throughout the study. Body weight of the offspring was determined on PNDs 1, 4, 7, 14, and 21 while weekly food intake was also calculated using weighing balance (Ohaus scale corporation, U.S.A).

Somatic landmarks. All animals were observed daily for alterations in behaviour or demeanour. Also detailed clinical examinations were conducted daily and also the following variables were observed. Postnatal somatic landmarks include day of 'Eye Opening'; day of 'Pinna Detachment' or 'ear opening'; day of 'Fur Appearance' and day of 'Incisor Eruption' (Almeida and Lemonica, 2007, Nunes et al., 2010). The days required for the appearance of these landmarks were recorded until all pups in the litters were positive for that developmental variable. The mean day of appearance of each of the above variables was calculated.

Anogenital distance and Anogenital index. The Anogenital Distance (AGD) was measured by using a vernier caliper capable of resolution to 0.1mm. It is defined as the distance from the anterior edge of the anus to the base of the genital tubercle. Anogenital distance (AGD) (millimeters) of all retained offspring/litter was measured by a trained technician blind to treatment group. To normalize for potential differences in body weight, the AGD index (AGD distance in mm/cube root of body weight) was calculated (Christiansen et al., 2009). The Anogenital Index (AGI) was measured on PND 1 and 4.

Statistics. Data obtained were expressed as mean ± standard error of mean (mean ± SEM). The significance of the results was evaluated using analysis of variance (ANOVA) and the means were compared using Tukey-Kramer Multiple comparison Test. Student's T- test was used to determine $P<0.05$ which was regarded as being statistically significant.

RESULTS

The food intake by the D21 rats was significantly reduced compared with that of the control rats at the 2nd and 3rd trimesters of pregnancy ($P<0.05$) (Fig 1.) while the maternal weights in the three trimesters were not statistically different ($P>0.05$) in the treated groups when compared with the control (Fig 2).

There was no significant difference in the food intakes of the treated dams from the control dams in the three weeks of lactation (Fig 3). There was also no significant difference in the mean body weight during lactation in rats administered CECS during pregnancy compared with that of the control in all three weeks (Fig 4). The weights of the rats at the recorded postpartum days were significantly greater than the corresponding pregravid weights in the D7 and D14 except in the Pretreatment rats while there was decrease in weight at postpartum day 1 when compared to the pregravid weight.

There was no significant difference in the length of gestation among the rats in the various groups. The litter sizes in the Pretreatment, D7 and D14 groups were significantly lower than the litter size of the control rats while the litter birth weights were significantly increased when compared to the control ($P<0.001$). Also the GD21 groups which were confirmed pregnant did not deliver any offspring (Table 1).

There was no significant difference in the pre-implantation loss in the treated rats when compared with the control rats, but there was a significant difference of the post implantation loss in D21 treated rats only when compared with the control (Table 2).

There was no significant difference in the food intake of the offspring at weeks 1 and 2. The food intake by the offspring of the Pretreatment and D14 CECS rats was significantly reduced when compared with that of the control rats ($P < 0.001$) at week 3. By week 4, there was an improvement in food intake of the offspring of the D14 CECS treated rats only when compared with the control ($P< 0.05$). There was also no significant difference at week 5 in the food intake of the offspring of the CECS treated rats when compared to the control (Fig 5).

Results show a significant increase in postnatal body weights at all periods (from birth to postnatal day 21) in the offspring of CECS rats compared with the offspring of the control rats. Pubertal,(postnatal day 61) body weights of the offspring of the CECS D7 and D14 rats only were increased significantly when compared with the offspring of the control rats (Fig 6).

There was a significant delay in the day of eye opening ($p< 0.001$) in the offspring of D7 and D14 treated rats when compared with the control. There was an earlier onset in the day of pinna detachment ($p< 0.001$) in the offspring of the all CECS treated rats when compared with the control. There was a significant delay in the day of appearance of fur ($p< 0.001$) in the offspring of the D14 CECS treated rats when compared to the control and there was an earlier onset in the day of appearance of fur of the offspring of the D7 ($P <0.05$) treated rats when compared with the control. There was an earlier onset in the day of incisor eruption ($p< 0.001$) in offspring of the Pretreatment group when compared with the control as well as a significant delay in the day of incisor eruption in the offspring of the D7 and D14 ($P <0.001$) treated rats when compared to the control (Table 3).

There was no statistical significance in the Anogenital Index on postnatal day 1 in the offspring of the CECS treated rats when compared with the control. On postnatal day 4 the Anogenital Index of the offspring from the pretreatment and D7 rats were significantly increased ($p< 0.001$) when compared with the offspring of the control rats while the D14 offspring had significantly reduced anogenital index when compared with the control (Fig 7).

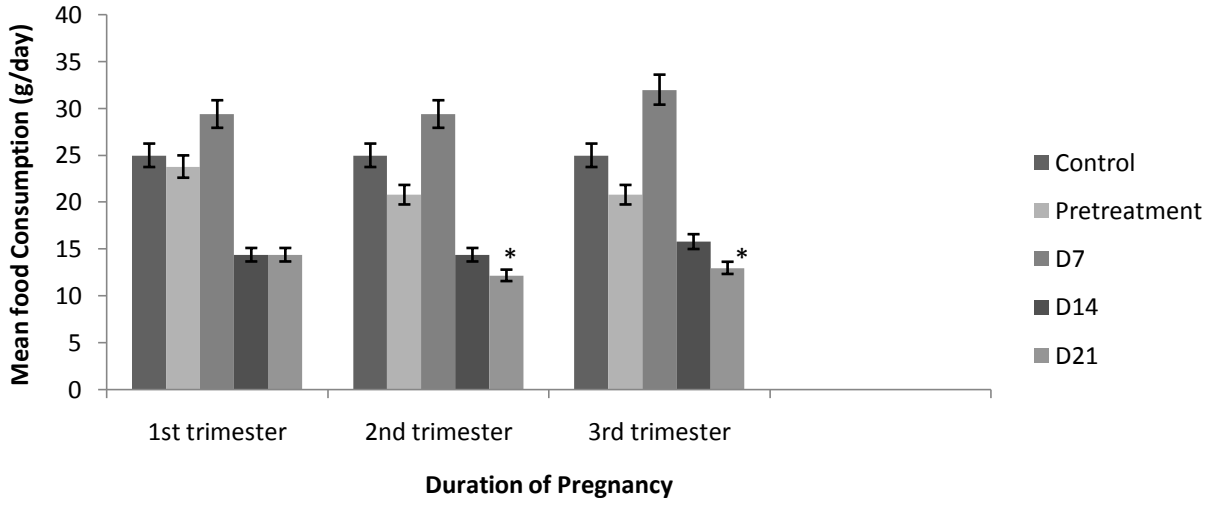

Fig 1. Maternal food consumption during pregnancy. Values expressed as Mean ± S.E.M. Values significantly different from control ($P<0.05$); n=5

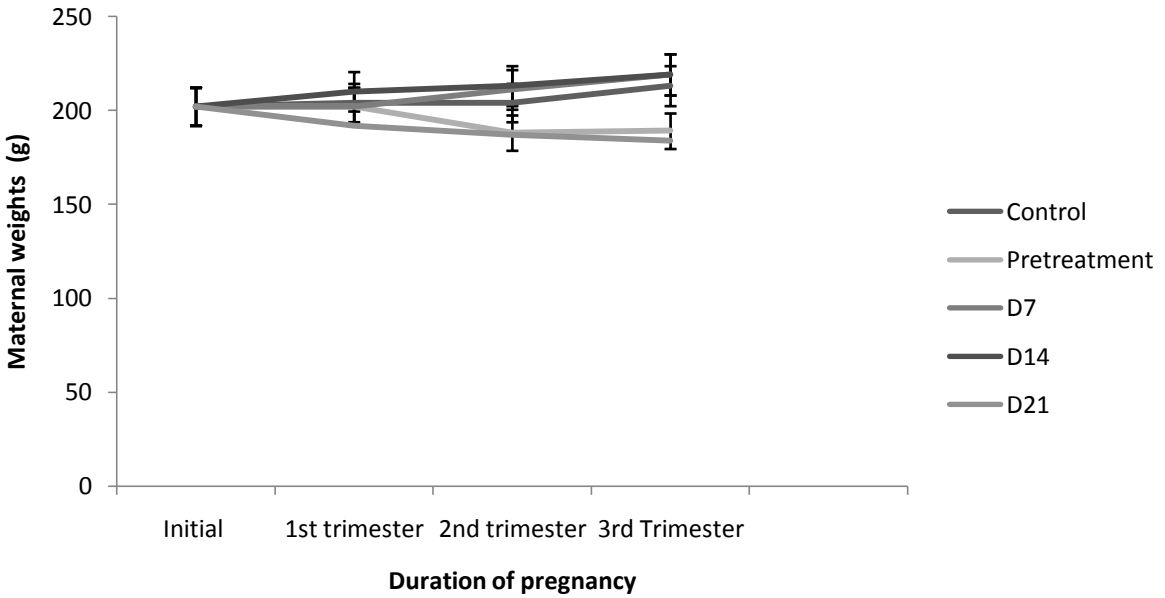

Fig 2. Maternal body weight during the three trimesters of pregnancy. Values expressed as Mean ± S.E.M, (P>0.05) compared to control; n=5

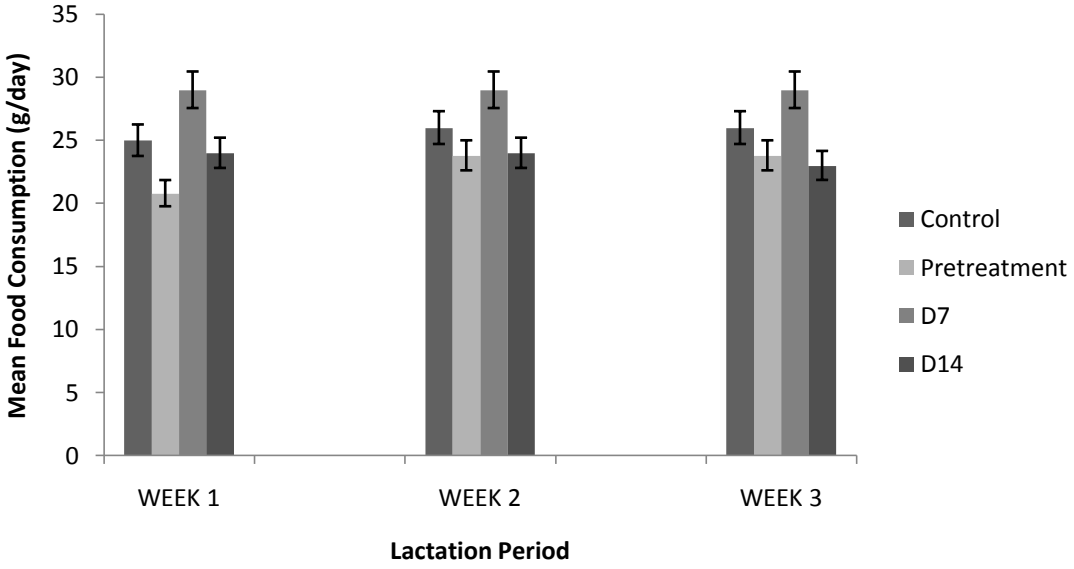

Fig 3. Maternal food consumption during lactation. Values expressed as Mean ± S.E.M. Values significantly different from control (P<0.05); n=5

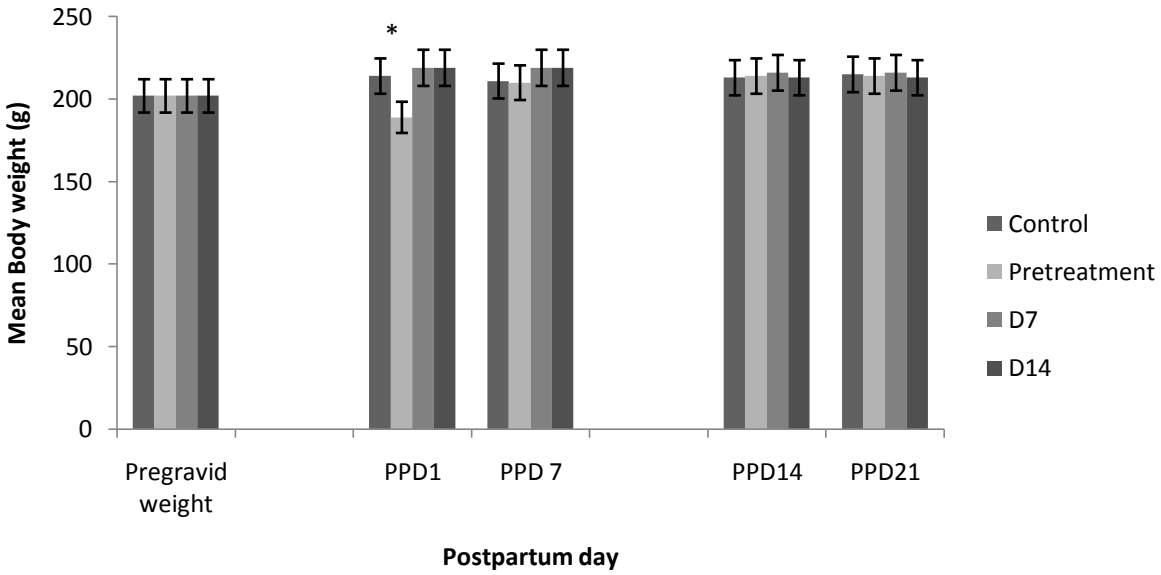

Fig 4.Maternal body weight during lactation (postpartum period). Values expressed as Mean ± S.E.M. n=5/group, PPD-postpartum day

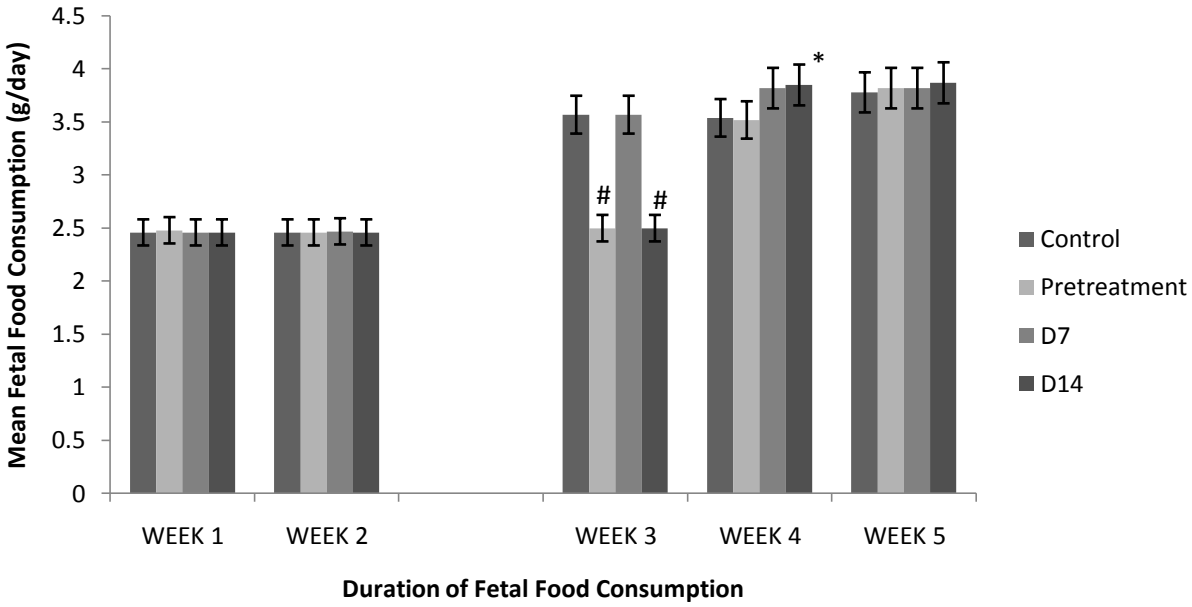

Fig 5. Postnatal food consumption of offspring. Values expressed as Mean ± S.E.M. Values significantly different from control (# P<0.001, *P<0.05); n= 45

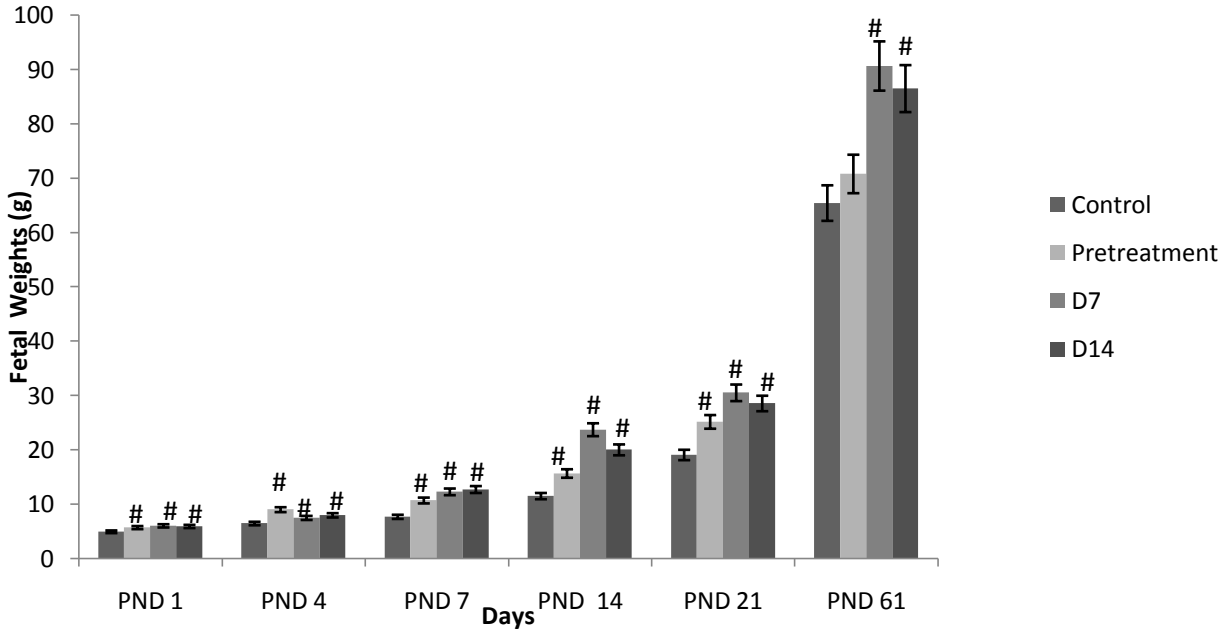

Fig 6. Postnatal weights of offspring. Values expressed as Mean ± S.E.M. Values significantly different from control (# P<0.001); n= 45

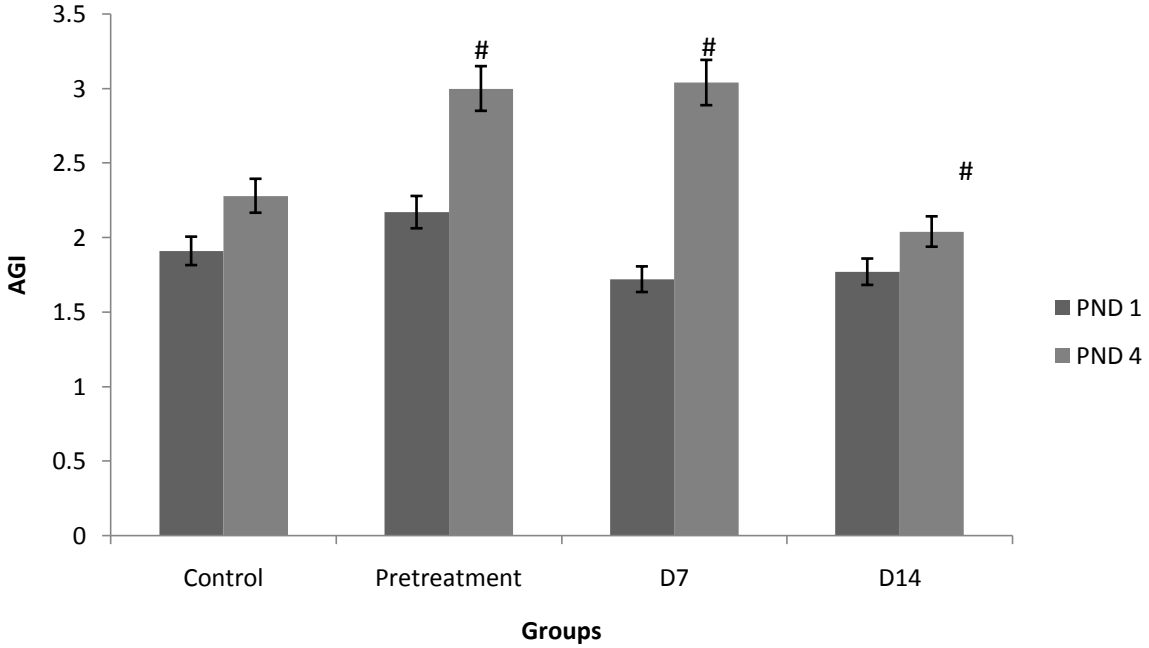

Fig 7. Anogenital Index (AGI) of the offspring .Values expressed as Mean ± S.E.M. Values significantly different from control (#P<0.001); n= 45

Table 1. Gestational length, litter size and litter birth weights following administration of CECS to groups

Groups	Length of gestation (days)	Litter size (n)	Litter birth weight (g)
Control	21.80±0.20	9.67±0.60	4.93±0.04
Pretreatment	20.40±0.24	7.5±0.05*	5.70±0.05#
D7	19.0±0.71	7.5±0.76*	6.02±0.08#
D14	21.60±1.21	5.5±0.88*	5.01±0.05#
D21	0	0	0

N=5 each. Values are expressed as Mean ± SEM, * = P<0.05, #P< 0.001)

Table 2. Pre- and post- Implantation index following administration of the chloroform extract of Carica papaya seed (CECS)

Groups	Pre- implantation Loss	Post-implantaion Loss
Control	1.2 ± 0.5	0.50± 0.60
Pretreatment	1.4 ± 0.5	0.65±0.69
D7	1.4 ± 0.5	0.58 ±0.60
D14	1.0 ± 0.5	0.59 ±0.09
D21	1.8±0.6	2.0±0.3#

N=5 each. Values are expressed as Mean ± SEM, (#P< 0.001)

Table 3. Age at somatic landmark attainment following administration of the chloroform extract of Carica papaya seed (CECS) during pregnancy

Groups	Day of Eye Opening	Day of Pinna Detachment	Day of Fur Appearance	Incisor Eruption
Control	13.78±0.09	18.00±0.00	7.00±0.00	7.00±0.04
Pretreatment	14.00±0.00	15.00±0.00#	7.00±0.00	6.49±0.08#
D7	15.49±0.08#	16.69±0.07#	6.87±0.05*	7.49±0.08#
D14	15.80±0.04#	17.00±0.00#	7.89±0.05#	7.78±0.11#

Values are expressed as Mean ± SEM, N= 45/group, * = P<0.05, # P< 0.001)

DISCUSSION

Studies have revealed that the epicarp, endocarp, seeds and leaves of *Carica papaya* have antifertility properties although the epicarp (skin) of the fruit contains more latex compared to the other parts of the fruit (Anuar et al., 2008). This implies that the solvent used for extraction does not determine its antifertility properties. For commercial purposes, papaya latex is harvested from fully-grown but unripe fruit. Ripe papaya contains less latex compared to green papaya possibly due to cessation of function or breakdown with age of the latex-producing cells (laticifers) (OECD, 2005).

Up-to-date traditional beliefs and practices still make analyzing physiological effect of *Carica papaya* of considerable interest especially in pregnancy states. Traditionally, in some parts of the World the use of green papaya to treat skin problems but its prohibition for consumption during pregnancy is obtainable (Anuar et al., 2008) while in some areas like Nigeria, papaya is eaten and used for medicinal purposes even in pregnancy.

In this study, administration of the chloroform extract of *Carica papaya* seed from ripe fruits at the first and second trimesters of pregnancy did not result in abortions in the treatment groups. According to (Anuar et al., 2008), most of the studies that had reported abortions used different parts e.g., pulp and seeds of unripe (green) papaya. However, in this study, the group administered the extract throughout pregnancy (D21) manifested abortificient properties which led to the zero fetal births. This is different from the study by (Raji et al., 2005) probably because the extract was administered to the animals two weeks before mating and throughout pregnancy and not when pregnancy was already established.

The D21 group had significantly reduced implantation sites which are similar to Oderinde et al., (2002) and Raji et al., (2005). The pre-implantation loss rate evaluates blastocyst implantation in the uterus, while post-implantation loss rate establishes correlation between the number of implanted blastocysts and those that have not developed (Chang et al., 2002, Almeida and Lemonica, 2007). In this study, the absence of statistical difference in the pre-implantation loss between the control and the CECS dosed groups is an indication that the implantation of the blastocysts was within normal range. This complements the findings of (Costa-Silva et al., 2007) on the effect of administration of *Carapa quianensis* seed oil during pregnancy in female wistar rats. Also the significant post-implantation loss in the CECS treated rats throughout pregnancy, (i.e. D21) connotes increased failure rate of embryo development and anti-pregnancy properties (Chang et al., 2002). The D21 groups, whose treatment continued throughout pregnancy, had the highest susceptibility of the extract to the embryos. Thus duration of extract exposure is important to consider in pregnancy and not the dosage alone.

There was no statistical difference in the length of gestation among the groups of rats administered CECS suggesting that the extract did not affect the length of gestation at the dose tested except for the D21 group. The litter sizes from the treated groups were decreased relative to the control rats. Since administration of CECS commenced on day 1 of pregnancy, the CECS may have interfered with the embryo-endometrial interaction at the time of blastocyst implantation, in a duration dependent manner, to decrease the implantation success rate and/ or decrease the number of foetuses resulting in the reduced litter size of CECS treated rats. Also foetal death in the GD 21 group is a manifestation of developmental fetotoxicity (Cavieres et al., 2002). It is thus tempting to propose that some sort of endocrine modulation is mediating the effects of the CECS extract on litter size. Also the survival rates of the offspring from the D21 groups can be said to be zero (Ulla et al., 2004).

The litter birth weights were significantly increased at PND 1 which was different from other studies. In a study by (Abdulazeez et al., 2009), who used fermented papaya seeds there was no significance in the litter birth weight. In another study, foetal weight was not affected when the papaya seed was administered before pregnancy but the foetal weight was adversely affected in the groups administered the extract during pregnancy (Raji et al., 2005). This is similar to (Oderinde et al., 2002) in a study which revealed that the aqueous extract of Carica papapya seed administered to female Sprague-Dawley rats at 100mg/kg body weight resulted in a significant decrease in foetal weight when compared with the control rats. Also at a dose of 800mg/kg body weight, surviving foetuses had stunted growths. These differences could be due to the fact that green papaya was used for the study as green papaya has been shown to reduce implantation sites (Schmidt 1995, Adebiyi et al., 2002). Green papaya has also been used to achieve abortion (Tiwari et al., 1992). Adaikan and Adebiyi, (2004) found that crude papaya latex and its proteinases such as papain and chymopapain are strong uterine contractants, explaining the abortifacient properties of papaya. Consumption of large quantities of unripe papaya fruit and subsequent ingestion of papaya latex can cause uncontrolled uterine contractions leading to abortion depending on the estrogen levels in the tissues that could be due to uterotonic effects of combination of enzymes, alkaloids and other substances instead of the pure papain itself (Cherian, 2000).). In this study increase in body weight continued up to the age of puberty despite the fluctuations in feed intake in the offspring.

Body weight and food consumption are sensitive indicators of toxicity. Maternal body weight and food consumption which are sensitive indicators of toxicity after exposure to a toxic substance did not show any adverse effect of treatment during pregnancy and lactation, except in the D21 CECS treated rats revealing the toxic effect of the substances at that duration of exposure.

Postnatal developmental alteration detected in the present study was as a result of substance produced delay by the treatment on the day of eye opening, pinna detachment, appearance of fur, as well as incisor eruption. The delays revealed that somatic development was impaired in the male pups of the CECS treated rats.

In utero exposure to CECS affected male fetal anogenital index. There was a significant increase in the anogenital index in male offspring exposed to CECS Pretreatment and D7 groups. This increase was about twice the index seen in the female anogenital index, revealing that the extract did not affect the offspring at the duration they were exposed to, but the D14 group showed a reduction in AGI, indicating possible future infertility problems.

Our study revealed that *Carica papaya* seed from ripe fruits are safer in pregnancy. Despite the abortifacient property of the *Carica papaya* seed, it is still in use in folklore medicine during pregnancy. Some of the reasons proffered for its abortifacient properties include the fact that according to the Indian traditional beliefs, especially among the Karnataka community, banana, papaya, jackfruit, pineapple and all unripe fruits and seed are perceived as 'hot' food and considered to be harmful for pregnant women (Adebiyi et al., 2002, Tiwari et al., 1992). Traditional beliefs and practices regarding papaya have thus led to several studies. What is now known is that traditionally, green or unripe papaya has been used to achieve abortion (Tiwari et al., 1992, Adaikan and Adebiyi, 2004). According to Anuar et al., (2008), the scientific claims of the abortifacient properties of *Carica papaya* plant have been investigated to be from the unripe (green) papaya. The concern however from our study is why the D21 treated animals still exhibited abortifacient properties despite the ripeness of the fruits used putting into cognisance the difference between semi-ripe and ripe fruits. These may not be farfetched from the other constituents of the papaya seed. These are however questions begging for answers and studies are on-going in our laboratory to unravel this.

In conclusion, the time window of pregnancy at which the chloroform extract of *Carica papaya* seed (CECS) exhibited an effect, which was from the second trimester of pregnancy was discovered in the study as there were no fetuses recorded in the third trimester. Since traditional medicine supports the use of *Carica papaya* seed in pregnancy, advocacy should be to consume seeds and fruits which are ripe, however, with caution.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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