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EXPERIMENTAL EVALUATION OF THE IMPACT OF MATERNAL CONSUMPTION OF AQUEOUS LEAF EXTRACT OF *HYBANTHUS ENNEASPERMUS* ON PREGNANCY IN *SPRAGUE DAWLEY* RATS

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Abstract

The impact of aqueous leaf extract of *Hybanthus enneaspermus* (HEaq) on pregnancy factors and litter survival was investigated in *Sprague Dawley* (SD) rat. Control group received distilled water while the test group received 2g/kg body weight of HEaq orally. Blood samples were collected on days one and twenty of pregnancy for total blood count, serum thyroid hormone, thyroid stimulating hormone (TSH) and thyrotropin releasing hormone (TRH) assay. Half the number of rats in each group was sacrificed on day nineteen of pregnancy and the placenta and foetus were removed and weighed. The second half carried their pregnancy to term. Number and weights of litter were recorded at birth and the litter were also subjected to righting reflex test. Post-natal survival rate was determined for each group while effect of HEaq was also examined in-vivo on the activities of pregnant myometrial muscle. HEaq significantly decreased ($p<0.05$) foetal weight, placenta weight, foetal growth and survival, number and weights of litter at birth, maternal serum triiodotyroxine T_3 and TSH level. Mean corpuscular haemoglobin, white blood cell count, platelet count and lipid profile were significantly increased ($P<0.05$). HEaq increased the frequency and percentage contraction of gravid myometrial muscle in a dose dependent manner. Maternal consumption of aqueous leaf extract of *Hybanthus enneaspermus* adversely affected pregnancy and development of the foetus, as it precipitated resorption of developing foetus and reduced size and weight of litter at term.

Key words: *Hybanthus enneaspermus* extract; Haematology; Pregnancy; Birth-weight; Thyroid hormones; Uterine contraction.

Introduction

The use of medicinal plants and their derived products for health related purposes is on the increase worldwide. Medicinal plants products are desirable as they are part of our daily diet eaten as vegetables or processed and taken as herbal tea. The advocacy for incorporation of Traditional medical practise into health care delivery towards actualization of the millennium development goals requires a framework. The framework will include among other things investigation, evaluation and monitoring of the various practises towards ascertaining the safety, beneficial and detrimental effects. This will also help towards improving on those practises that are beneficial while discouraging those ones that proof detrimental to the health of the people.

In southwest Nigeria, *Hybanthus enneaspermus* locally known as '*Abiwere'* (by interpretation; 'leaf that makes delivery less laborious') is one of the major medicinal plants employed by the Traditional Birth Attendants (TBAs) in the care of pregnant women. *Hybanthus enneaspermus* is a perennial shrub growing in the rainforest area in western Nigeria. The leaf extract of the plant has been reported to have anticonvulsant and free radical scavenging activities (Hemalatha 2003), antiplasmodial activities (Weniger *et al*., 2004), anti arthritic activities (Tripathy *et al*., 2009), antimicrobial and antifungal effect of the leaf extract of the plant against some urinary tract pathogens (Sahoo *et al.,* 2006, Awobajo *et al.,* 2009a) hypoglycaemic activities (Awobajo *et al*., 2010) and wide oral dose safety margin (Awobajo *et al.,* 2009b). Recent report on the active constituent of the leave extract of *Hybanthus enneaspermus* revealed the presence of nicotine alkaloid (Amutha *et al*., 2011). There are however, dearth of scientific information on the possible effects of oral consumption of the leaf extract during pregnancy on maternal physiology and the pregnancy outcome. This study was therefore designed to investigate the effects of the maternal consumption of the leaf extract of *Hybanthus enneaspermus* on pregnancy related parameters and thyroid activities within Sprague Dawley rat.

Material and Methods

Plant preparation and extraction

A survey was carried out with the aid of a questionnaire administered on some Traditional medical practitioner

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living around Ijebu North local government area of Ogun state Nigeria, to identify the common medicinal plants used during antenatal care. The purpose of the survey was well explained to participating TBAs and Herb sellers (Eleweomo) before they agreed to participate in the review of common herbs used in ante natal care. *Hybanthus enneaspermus* emerged as the most common (100%). Identification of *Hybanthus enneaspermus* plant used by Traditional Birth Attendants was done by Late Chief Ogunsipe from Awa- Ijebu. The plants were harvested from a farmland in Ijebu-Ode, southwest Nigeria and botanical identification carried out at Forestry Research Institute of Nigeria (FRIN) Ibadan, Nigeria. A voucher number FHI NO.108226 was deposited at the FRIN herbarium. The leaves were chopped into pieces and air-dried in a dust-free environment. Aqueous extraction of the leave was carried out using Soxhlet extractor as earlier described (Awobajor *et al.,* 2009b). The extract yield was lyophilized and kept at -4^0 C until used. The presence and concentration of some macro and microelements in the leaf extract was determined using atomic absorption spectrometry method (Omolo *et al.,* 1997).

Animal grouping, treatment and data collection

The female *Sprague Dawley* rats used for this experiment weighed between 170–180 gram. They were divided into two groups; control and HEaq groups with twelve rats per group. The animals were procured from rat colony, College of Medicine, University of Lagos. In each rat, pregnancy was established by the presence of sperm cell in the vagina smear after overnight cohabitation with male of proven fertility (two females to one male). Animals were cohabited at proestrus after carefully monitoring the estrous cycle. Day one of pregnancy was taken as the day sperm cells were first recorded in the vagina smear. Blood samples (1.5 ml) were collected via peri-orbital venous sinus (Hoff 2000) on day one of pregnancy for automated blood cell count Sysmex KX-21N, Code no 461-2265-5) USA, while the plasma was used to for the analysis of the lipid profile (High density lipoprotein (HDL), Low density lipoprotein (LDL), Triglyceride (TGL) and Total cholesterol (TChol), and thyroid hormones (Triiodothyronine (T3), Thyroxine (T4) and Thyrotropin releasing hormone (TRH), Thyroid stimulating hormone (TSH)) using Randox assay kit. The extract treated rats were thereafter administered 2 g/kg body weight of HEaq using oral dosing needle, while the control received equal volume of distilled water. The animals were housed in clean cages under twelve hours light and twelve hours darkness while rat chow and clean tap water was provided *ad libitum*. Maternal gestational weights were recorded weekly. Number of viable implants and resorption was determined on day nineteen of pregnancy. The rats were sacrificed by cervical dislocation and laparactomised while the uterus were carefully opened up and number of implants counted as well as resorption site that appears vascularised without the presence of growing foetus.

The foetuses were carefully removed along with the placenta and weighed separately free of amniotic fluid. Blood samples (1.5ml) was withdrawn from peri-orbital venous sinus (Hoff 2000) from the remaining pregnant rats in both control and HEaq groups on day twenty of pregnancy using heparinised capillary tube for whole blood analysis while the serum was used for lipid profile and thyroid hormones assay. All animals were fasted for twelve hours before collection of blood samples. The rest of the animals were left to carry the pregnancy to term, and the number and weights of litter delivered was recorded few hours after delivery. The litter responses to righting reflex test (Vorhees *et al.,* 1994) were monitored for the first seven days after delivery and the postnatal survival rate monitored and recorded after fourteen days.

Uterine muscle contraction

Uterine muscle from two weeks old pregnant rats that were not pre-treated with extract were used for the study on myometrial muscle response to HEaq extract. The animals were sacrificed by cervical dislocation, the abdomen carefully opened up and the two horns of the uterus removed and freed of mesenteric fat. Piece of uterine tissue (2mm) was removed and mounted in 50 ml organ bath connected to a force transducer (Grass Model FT03), coupled to a Grass Model 7D polygraph for recording of isometric tension (Elias *et al.,* 2007). The transducer was pre-calibrated to give a 2 cm deflection for every 1gram force. The organ bath was filled with physiological salt solution, aerated with oxygen (5% CO_2), and maintained at 37^o C. The tissue was allowed to equilibrate for 60-90 minutes, spontaneous contraction was recorded and the effects of graded concentration of freshly prepared HEaq were observed on the contractility of the uterine muscle.

Analysis of results

Results were presented in tables as Mean ± SEM and analysed using ANOVA. Level of significance was placed at $p < 0.05$, while bar charts were used for graphical presentation. The rules guiding animal experimentation and humane treatment of animals used in Biomedical research were observed

Results

The extract was found to contain high amount of iron (25.78%) as one of the major micro elements (Table 1). There was no significant difference in weight gained during pregnancy as well as gestational duration between control and extract treated rats. The number of viable foetus was significantly lowered ($p<0.05$) in HEaq treated rats compared with control. There was also a significant reduction ($p<0.05$) in maternal serum T_3 and TSH level with concomitant significant reduction ($p <0.05$) in placenta weight in HEaq treated rats (Table 2, Tab 4). Both litter size and weight were significantly reduced ($p < 0.05$) at delivery in HEaq treated rats (Table 2). The haematological parameters measured showed only a significant increase ($p < 0.05$) in haemoglobin concentration and lymphocyte count (Table 3). All litter in both control and extract treated group of rats recorded 100% survival rate two weeks post natal period but with a significant ($p< 0.05$)

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difference in percentage change in righting reflex response time (Table 5). There was also a significant increase ($p<0.05$) increase in TGL, HDL, LDL and TChol. in HEaq extract treated rats compared to control. In vivo results of contraction of gravid uterine muscle also showed a concentration dependent increase in frequency of contraction with the highest percentage contraction recorded at 0.37 mg/ml final bath concentration

Table 1: Elemental detection in leaf of *Hybanthus enneaspermus* on a dry weight basis using atomic absorption spectrophotometry method

S/No	Type	Element	Quantity (mg/kg)
1	Macro	Calcium	54.67
2		Magnesium	19.04
3		Zinc	13.87
4		Sodium	8.12
5		Potassium	0.23
6	Micro	Iron	25.78
7		Manganese	4.98
8		Copper	0.49
9		Argon	0.16
10		Lead	0.06
11		Chromium	0.06
13		Cardium	Not detected
14		Nikel	Not detected

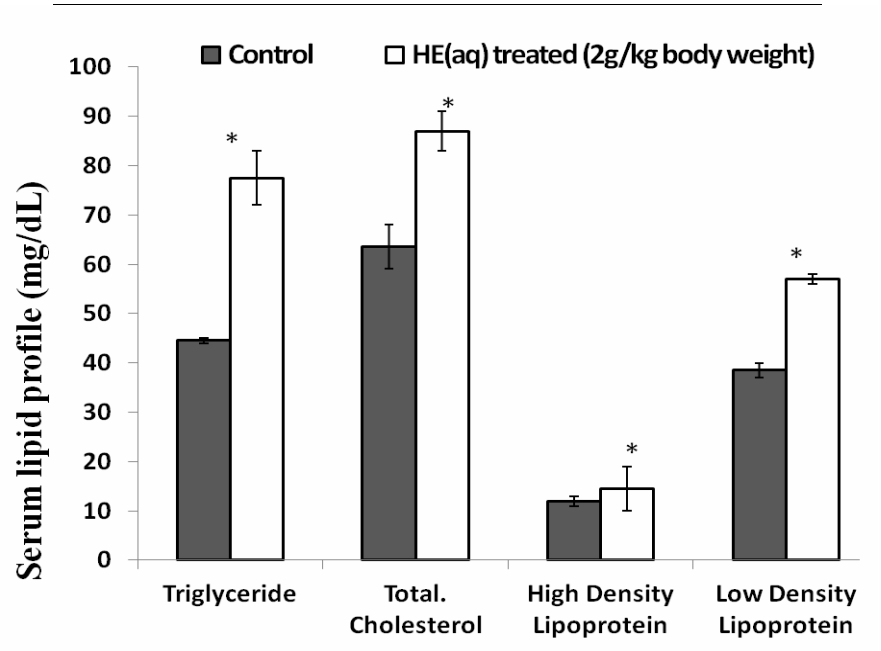

Figure 1: Effect of oral administration of aqueous leaf extract of *Hybanthus enneaspermus* on serum lipid profile in pregnant rats * = Significantly different compared with control ($p < 0.05$)

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Table 2: Effects of oral administration of aqueous leaf extract of *Hybanthus enneaspermus* on number of viable foetus resorption sites at 19th day of pregnancy, maternal weight gained during pregnancy, duration of pregnancy, litter size and litter weight at delivery

Group	Weight gain during pregnancy (g)	At 19th day of pregnancy				Gestational duration (days)	Day of delivery	
		Foetal weight(g)	Placenta weight(g)	Number of Resorption site	Number of viable foetus		Litter size (n)	Litter weight (g)
Control	40.00 ± 5.77	2.23 ± 0.07	0.53 ± 0.04	0.00 ± 0.00	10.50 ± 0.50	21.00 ± 0.32	10.50 ± 0.50	4.51 ± 0.22
HEaq	40.00 ± 5.52	1.72 ± 0.04*	0.41 ± 0.03*	1.50 ± 0.05 $^{\beta}$	8.00 ± 0.41*	21.00 ± 0.32	8.00 ± 0.50*	3.68 ± 0.21*

- Significantly reduced ($p \leq 0.05$) compared with control. $^{\beta}$ significantly increased ($p \leq 0.05$) compared with control

Table 3: Effect of oral administration of aqueous leaf extract of *Hybanthus enneaspermus* from day one until day twenty-one of pregnancy on haematological parameters

Parameters	Initial reading (Day 1)	At 20th day of pregnancy	
		Control	HEaq
Red Blood cell count(x10^{12}/L)	6.42 ± 0.10	6.46 ± 0.10	6.45 ± 0.18
Haemoglobin concentration (g/dL)	13.23 ± 0.06	13.50 ± 0.06	14.18 ± 0.06*
Packed cell volume (%)	33.67 ± 0.47	35.00 ± 0.35*	36.97 ± 1.00*
MCV (fl)	52.44 ± 0.58	54.18 ± 2.48*	57.94 ± 0.35*
MCH (pg)	20.61 ± 0.22	20.90 ± 0.50	21.96 ± 0.48*
White blood cell count(x10^{9}/L)	7.60 ± 1.31	8.80 ± 1.20	8.85 ± 1.40*
Lymphocyte count (x10^{9}/L)	6.83 ± 0.30	8.20 ± 0.62*	7.80 ± 0.50*
Granulocyte (x10^{9}/L)	0.30 ± 0.20	0.30 ± 0.20	0.43 ± 0.10*
Monocytes (x10^{9}/L)	0.30 ± 0.20	0.30 ± 0.20	0.67 ± 0.30
Platelet count (x10^{9}/L)	89.33 ± 12.71	173.50±20.00*	197.00±21.73*

* Significantly increased ($p \leq 0.05$) compared to day one of pregnancy,

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Table 4: Effect of oral administration of aqueous leaf extract of *Hybanthus enneaspermus* to pregnant rats on serum T_3, T_4, TSH and TRH

Group	T_3 (nmol/l)	% Decrease in T_3	T_4 (nmol/l)	% Decrease in T_4	T_4/T_3 ratio	TSH (µIu/ml)	TRH (Iu/ml)
Control	2.76 ± 0.08		74.00 ± 1.17		26.85 ± 0.57	3.35 ± 0.64	26.55 ± 3.61
HEaq	1.77 ± 0.09*	1.34± 0.22#	70.14 ± 0.43	5.17 ± 1.12	40.14 ± 2.54†	1.12 ± 0.26*	26.70 ± 7.35

* Significantly lowered compare to the control ($p \leq 0.05$)
†Significantly increased compared to control ($p \leq 0.05$)
Significantly lowered compared to T_4 level

Table 5: Effect of oral administration of aqueous leaf extract of *Hybanthus enneaspermus* to pregnant rats on righting reflex test on the litter delivered

Group	Righting reflex test (second)		% Change in reflex response	Survival Rate on the 7th day (%) $\{\frac{\text{litters number on test day}}{\text{Litter number at birth}} x100\}$	Survival Rate on the 14th day (%) $\{\frac{\text{litters number on test day}}{\text{Litter number at birth}} x100\}$
	Day 1	Day 7			
Control	1.19 ± 0.10	0.43 ± 0.03	184.57 ± 37.86	100	100
HEaq	1.21 ± 0.08	0.45 ± 0.03	169.14 ± 32.58*	100	100

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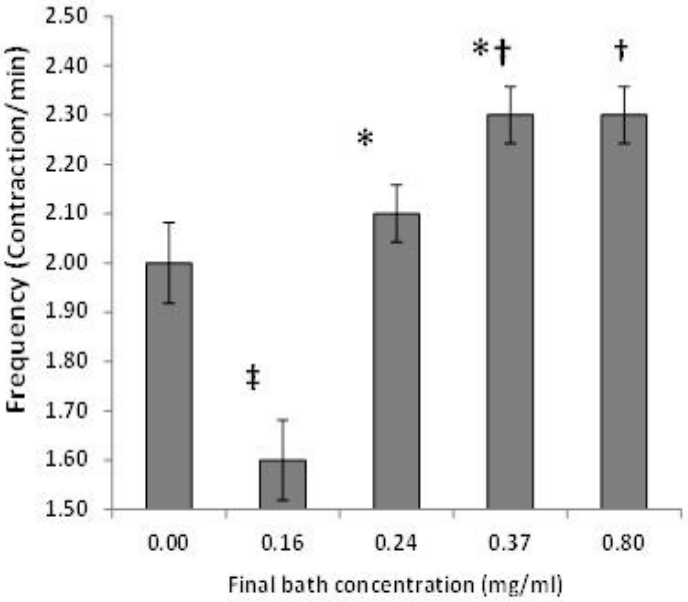

Fig. 2a: Effects of graded concentration of HEaq on frequency of spontaneous contraction of myometrial strips from two weeks old pregnant rats

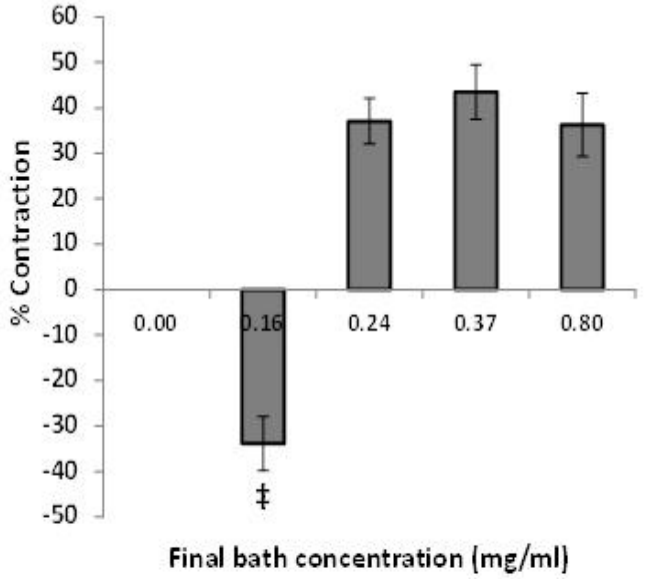

Fig. 2b: Effects of graded concentration of HEaq on spontaneous isometric contraction y of uterine strips from two weeks old pregnant rat

* Significantly increased compared to previous value
† Significantly increasedcompared to intial value
‡ Significantly reduced compared to initial spontaneous contraction
Note: Final bath concentration was a cumulative concentration of the extract in 50ml organ bath

Fig. 2: The effects of HEaq on uterine contraction in two weeks pregnant rats

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Discussion

Medicinal plants have different applications and effects on the body system. Some of the effects are desirable while others are deleterious to the normal body functions. Contrary to the folkloric use of the leaf of *Hybanthus enneaspermus* as blood tonic, the results of this study revealed no significant effect at the selected dose on the red blood cell count in the pregnant rats within the gestational period (Table 3). However, there was a significant increase in haemoglobin concentration and white blood cell count in the pregnant rats. Atomic absorption spectrophometry analysis of the leaf extract of the plant revealed the presence of iron at a concentration of 25.78 mg/kg of the dry weight of the powdered leaf (Table 1). Although, iron absorption from the gut is also subject to several other factors however, this additional supply of iron will increase the available pool for the synthesis of haemoglobin. The increase in white blood cell count recorded in this experiment was due to the significant increase recorded in lymphocyte count in the extract treated rats. Antimicrobial and anti fungal activities of the leaf extract especially against urinary tract infection causing organism have been reported (Sahoo et al., 2006, Awobajo et al., 2009a).

In this study, oral administration of leaf extract of *Hybanthus enneaspermus* did not interfere with maternal weight gain and the length of gestation. However, foetal and placenta weight at day nineteen of pregnancy as well as litter birth weights were significantly reduced. The Foetus gained over half of their weight between the nineteenth and twentieth day of pregnancy (Tab 2). Placenta growth is regulated by the nutrient availability (Lumey 1998) as well as maternal thyroid hormone level (Ekins 1985). Hypoglycaemic potentials of aqueous leaf extract of *Hybanthus enneaspermus* have been reported (Awobajoi et al., 2010, Patel et al., 2011). Thus, there is the possibility of the hypoglycaemic effect of the extract, potentiating maternal accumulation of energy inform of fat to the detriment of the developing foetus. This was evident in the similar weight gain recorded between the extract treated and the control rats despite the significant reduction in the number as well as weights of the litter delivered at birth.

The primary functions of the placenta is to provide an immunological barrier between foetus and mother, mediate the transfer of gases, water, ions and nutrients and to secrete a vast array of hormones involved in cellular signaling (Jansson and Powell 2007). Foetal nutrition and growth is closely related to placenta functions with increased placenta weight initiating corresponding foetal weight gain (Sanin et al,2001). The reduction in the placenta weight recorded in this study may be a sign of disruption of the histomorphometry of the placenta that also translate into a significant reduction in the litter weights at term (Tab 3). Recent report has indicated the active presence of nicotine alkaloid in the extract of *Hybanthus enneaspermus* (Amutha et al., 2011). Maternal exposure to nicotine has been reported to stimulate significant alteration of histo-chemical architecture of the placenta enough to affect its functions (El-Meligy 2007).

Pregnancy period requires the lowering of the electrical activities of the uterine muscle by a way of increasing the threshold of excitation of the myometrium as well as reducing the estrogenic receptor activities known to be responsible for the contraction; a state of quiescent. This state is achieved by increased progesterone level in circulation, which inhibits the activities of estrogens and its receptors. The use of any substance that could trigger uterine contraction before the completion of the gestational period could trigger premature uterine contraction with consequent abortion of the growing foetus. *In-vitro* administration of HEaq produced a dose dependent increase in frequency and force of contraction of the two weeks old pregnant myometrial muscle (Figure 3). Further work will however be required to identify the uterotonic substance present in the extract as well as its mode of action and the receptors involved in the observed increased in-vivo contraction response to HEaq on two weeks old pregnant myometrial muscle.

Apart from nutrient availability, maternal thyroxine level is also essential for normal development and neurological maturation of the foetus before the foetal thyroid becomes functional (Pharoah et al., 1984). Thyroid function is controlled partly by changes in maternal energy intake (Risss and Madsen 1985) or iodine status (Elton et al., 2001). Thyroid hormone is important in the development of the nervous system with irreparable damage consequent to the deficiency during uterine life (Heyerdahl 1991, Berbel et al., 2009). Maternal thyroid activity is increased during pregnancy (de Escobar et al., 2007) due to increased production of thyroid stimulating hormone (TSH) or to a physiological production of placental thyrotrophic substances (Omolo et al., 1997) with increased requirement for iodine. Oral administration of HEaq to pregnant rats precipitated a significant reduction in maternal T_3 and TSH level, placental weight and litter birth weights compared to the untreated control rats. The T_4:T_3 ratio was significantly elevated in the extract treated group giving an indication of possible inhibition of iodothyronine 5'deiodinase responsible for conversion of T_4 to T_3, which is the metabolically-active thyroid hormone influencing gene expression required for maximum biological activity (Sidibeel 2007). The prevalence of hypothyroidism in sub-Saharan Africa is 8.8% (Frumess and Larsen 1975) showing that hypothyroidism is still a public health concern in this part of the world, especially where maternal mortality rate is still very high. The prevalence of hypothyroidism in this part of the world will therefore, be worsened by indiscriminate consumption of such medicinal plants that have anti-thyroid activities, especially when used during pregnancy. Increased maternal thyroxine level also stimulates placenta growth (Spencer and Robinson 1993). Therefore, the reduction in maternal thyroid hormone level especially the markedly reduced T_3 (Tab. 3.) level contributed to the significant reduction in placenta weight and thus the recorded decreased birth weights (Table 1). This result corroborates previous report by Wrutniak and Cabello (1983).

All litter born to control and the HEaq treated rats recorded a decrease in the righting reflex time between first and seventh day of extra uterine life; an indication that there was no significant damage to the nervous system. However, the degree of improvement in this test after postnatal day seven was lowered significantly in litter born to HEaq treated rats when compared to the control (Tab.4). This result gave an indication of possible adverse effect of the in-utero exposure of the litters to the HEaq extract on the development of their nervous system. There will however be need for further investigation to confirm the reason for the delayed response.

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Conclusion

From the results of this study, it is evident that oral consumption of aqueous leaf extracts of *Hybanthus enneaspermus* by pregnant rats adversely affected maternal lipid profile, impacted negatively on the sustainability and development of the foetus and also produced a dose dependent contraction of gravid uterine muscle.

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