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Dioscorea prehensilis Ameliorates the Features of Preeclampsia in L-N^G-Nitroarginine Methyl Ester (L-NAME) Preeclamptic Model

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ABSTRACT

Preeclampsia is a major cause of maternal and perinatal mortality and morbidity. Sadly, effective therapeutic strategies do not exist up to now for preeclampsia but antiplatelet agents, low dose aspirin in particular is considered as primary prevention in high risk patients. *Dioscorea prehensilis* (DP) belonging to the family Dioscoreaceae is used among locals in the treatment of severe inflammatory conditions. This study is aimed at studying the effect of DP on the components of preeclamptic syndrome in a rat model. The rhizome of DP was extracted by maceration in 80% methanol. Preeclampsia was induced by chronic inhibition of nitric oxide synthesis by L-NAME (50 mg/kg/day, orally) in pregnant rats. DP was administered at a dose of 500 mg/kg/day orally. Systolic blood pressure was measured using tail cuff method on day 20. Albumin/creatinine ratio in spot urine was assessed using standard protocol on day 19; plasma nitric oxide was quantified using commercially available kits on day 20 while histological analysis was done using standard procedures. The extract reduced the systolic blood pressure significantly when compared to the preeclamptic control (130.38±4.43 versus 92.28±23.33mmHg) and not significantly different from the normal control (108±3.41 mmHg). The mean albumin/creatinine ratio was also reduced compared to the preeclamptic control though not significantly. Histological examination of the placenta shows that the extract reduced the severity of hypoplastic villi and intervillus congestion compared to the untreated group. In conclusion, DP ameliorates preeclampsia and may serve as a source of bioactive agents for the management of preeclampsia.

Keywords: *Dioscorea prehensilis*, Preeclampsia, L-NAME, Rhizome.

Introduction

Hypertensive disorders of pregnancy complicate roughly 5–10% of pregnancies.[1] A World Health Organization review identified hypertension as the single leading cause of maternal mortality in industrialized countries, accounting for 16% of deaths.[2] Hypertension during pregnancy has been classified into four categories namely; Preeclampsia –eclampsia, chronic hypertension (of any cause), chronic hypertension with superimposed preeclampsia and gestational hypertension.[3] Amongst these hypertensive disorders that complicate pregnancy, pre-eclampsia and eclampsia stand out as major causes of maternal and perinatal mortality and morbidity.[4]

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Sadly, effective therapeutic strategies do not exist up to now for preeclampsia.[5] This is because current therapies do not ameliorate the placental pathology nor alter the pathophysiology or natural history of preeclampsia hence it is a progressive disorder that will inevitably worsen if pregnancy continues and the only cure is termination of pregnancy/delivery of the fetus and placenta.[6] This indicated delivery of women with preeclampsia in order to prevent its progression is responsible for 15% of all preterm births.[7] Clearly, a simple and effective intervention is urgently needed.

Drugs have been sourced from nature including the use of plants, minerals, animals, microorganisms and synthesis *e.g.* more recently combinatorial chemistry.[8,9] Nonetheless, plants-based drug discovery appears to have been most successful and drugs from plant dominate the market, accounting for over 50% of drugs currently in the markets.[10] Also, a clear definition of pathophysiology and molecular targets which are largely unknown in preeclampsia are required if recent combinatorial chemistry are to be used. It is therefore, reasonable to focus on botanicals for a drug discovery effort targeting the pre-eclamptic syndrome.

Although the etiology and pathophysiolgy of preeclampsia is still not clearly understood, a marked reduction in placental blood flow that eventually manifests as defective placentation and placental hypoxia has been postulated.[6] Preeclampsia is also characterized by an inflammatory response after ischemia and reperfusion.[11] Prophylactic therapy with antiplatelet agents has been the subject of a large number of studies and systematic reviews.[12,13] Given the modest but significant

protective effect, low dose aspirin prophylaxis is considered as primary prevention for preeclampsia in women at high risk and if used should be initiated in the late first trimester.[14]

Dioscorea prehensilis, commonly called wild yam belongs to the family Dioscoreaceae. It is locally known in Hausa as Magurasa. It is used among the locals in the treatment of severe inflammatory conditions. A previous study on the antiplatelet aggregation property of the methanol extract of its rhizome found that the extract possesses a significant antiplatelet aggregation property, with an IC_{50} lower than that of aspirin (yet to be unpublished data). We hypothesized that this extract will ameliorate the symptoms associated with preeclampsia and this hypothesis is hereby investigated in this study.

Materials and Methods

Animals

Male and female Sprague – Dawley rats were purchased from the animal house of the Department of Pharmacology, Ahmadu Bello University, Zaria. The animals were housed under controlled conditions of temperature (23±2ºc), humidity (55 ± 10%) and lighting (12-h light/dark cycle) and provided with food and water *ad libitum*.

Animals were acclimated for 2 weeks after arrival. For mating, females were placed with males overnight and examined the next morning for the presence of sperm in a vaginal smear. The day sperm is detected was counted as gestational day 0. Pregnant animals were housed two per cage with access to water and feed *ad libitum.* All experiments were conducted according to the institution's guidelines for care and use of laboratory animals in research.

Preparation of Plants Materials and Extraction

The rhizome of *Dioscorea prehensilis* was collected in Sokoto in September 2015, identified and authenticated by a consultant taxonomist to the herbarium of Department of Pharmacognosy and Ethnomedicine, Faculty of Pharmaceutical sciences, Usmanu Danfodiyo University, Sokoto (Mallam Umar Galla). Voucher specimen has been deposited in the herbarium unit (PCG/UDUS/Dios/0002)**.** The plant material was then air dried under shade to constant weight and size reduced into fine powder using pestle and mortar. One hundred and forty (140) grams of the powdered material was then subjected to extraction by maceration using 450 mL of 80% methanol for 5 days. The resulting extract was evaporated to dryness using water bath controlled at 45ºC.

Acute Toxicity Studies

The acute oral toxicity study was done by the "Up and Down method" in healthy adult female albino rats according to OECD guidelines no 425.[15] A limit dose of 2000 mg/kg was used for the study.

Treatment regimen

The method of Richer *et al*[16] was adopted. On day 14 of pregnancy, the animals were randomized into 3 groups of 5 animals each. The first group received the vehicle distilled water (normal control), the second one was given L-NAME, 50 mg/kg/day, by gavage and vehicle). This is the pre-eclamptic control group. Group 3 received L-NAME (50 mg/kg/day, by gavage) alongside with 500 mg/kg/day of the methanol plant extract by gavage. This treatment continued until day 20 gestation. Gestation was terminated on day 20 with a caesarean session.

Blood Pressure Measurement

The blood pressure of each animal was taken on day 20 using the non-invasive tail cuff method (Ugobasil). Blood pressure was obtained five times and mean of each rat recorded.

Urine Collection and Assessment of Proteinuria

On day 19, each animal was housed in an individual metabolic cage for 3-4 hours to allow a spot urine collection for urinalysis. This was done in the absence of food to avoid contamination by fallen food particles or faeces. The albumin concentration of each urine sample was determined with the Bromocresol green assay. The assays were done according to the directions given in the kit by the manufacturer. The urine samples were analyzed for creatinine concentration using the alkaline picrate method which relies on the Jaffe reaction, in which the samples form a yellow /orange colour when treated with alkaline picrate.[17,18] The colour intensity (which is proportional to the creatinine concentration of the sample) was measured at a wavelength of 500 nm. The level of proteinuria was determined by calculating mg albumin/mmol of creatinine.

Sample Collection

On day 20, each animal was sacrificed as described below. Each animal was anaesthetized with chloroform. Blood samples were obtained by cardiac puncture and separated into serum, plasma and whole blood specimens. Thereafter, a laparotomy was performed to expose the uterine horns. The number of develop fetuses and their respective placenta were counted, removed and weighed. The heart, kidney and liver were rapidly removed and were suspended alongside the placenta in 10% buffered formalin for histological investigations.

Measurement of Total nitric oxide

Plasma nitric oxide (NO) levels were measured following the reduction of nitrate to nitrite by an improved Griess method, using a commercially available kit according to the manufacturer's protocol (Oxford Biomedical Research Inc. USA).

Histopathological Examinations

Collected organs (placenta, kidney, heart and liver) were immediately fixed in 10% buffered formaldehyde and left for at least 24 hours. The thickness of the placenta disc and gross macroscopic examination of the placenta tissue for areas of infarction were determined. Sections of the placenta membrane, placenta disc and umbilical cord each were made from each placenta tissue. All fixed tissues ((placenta, kidney, heart and liver) were dehydrated in an ascending series of alcohol, cleared in xylene and embedded in paraffin wax melting at 60^0C. Serial sections (5-µm thick) were mounted on 3-aminopropyl triethsilane- coated slides and dried for 24 hours at 37^0C.[19] The sections on the slides were deparaffinised, hydrated and stained with Mayer's hematoxylin and eosin dyes, dried and mounted.

Statistical analysis

All the data expressed are the mean ± standard deviation from the mean. The student *t-test* was used to compare the means and p value of less than or equal to 0.05 was considered significant. Graphpad prism (version 6) was the statistical package used.

Results and Discussion

Acute toxicity studies

At a limit dose of 2000 mg/kg, all the rats in the short and long-term observation survived. There was neither mortality nor sign of toxicity in all the treated animals. This implies that the LD_{50} is greater than 2000 mg/kg and relatively non-toxic.

Blood pressure measurements

The systolic blood pressure (SBP) measurements of each group taken on day 20 gestation were compared (Figure 1). There was a significant increase in SBP when the normal control was compared with the preeclamptic control (108.18 ± 3.41 versus 130.38 ± 4.43). The extract of DP caused a significant reduction in the SBP when compared to the preeclamptic control (preeclamptic control, 130.38 ± 4.43;.DP, 92.28 ± 23.33). When compared to the normal control however, the SBP were not significantly different.

Pup weight

The pup weights of all the groups were compared after 20 days gestation (Figure 2). The normal control group was significantly higher than the preeclamptic control group (5.14 ± 0.63 versus 3.21 ± 0.36). The plant extract did not show a significant difference in the pup weight when compared with the preeclamptic control. When compared with the normal control however, the pup weights were significantly lower.

Placenta weight

The placenta weights of all the groups were compared after 20 days gestation (Figure 3). There was no significant difference in the placenta weight of the normal, preeclamptic control and the treated group (0.95 ± 0.04, 0.91 ± 0.07 and 0.76 ± 0.21 respectively).

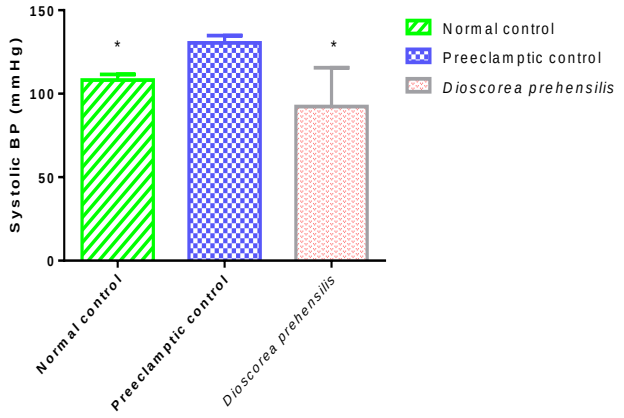


Figure 1: The systolic blood pressure of the normal control, Preeclamptic control and the plant extracts on days 19. Blood pressure was recorded non-invasively using the tail-cuff method. The data is expressed in millimeters of mercury (mmHg) and presented as mean ± SD. Bars with (*) above are significantly different ($p < 0.05$) from the preeclamptic control

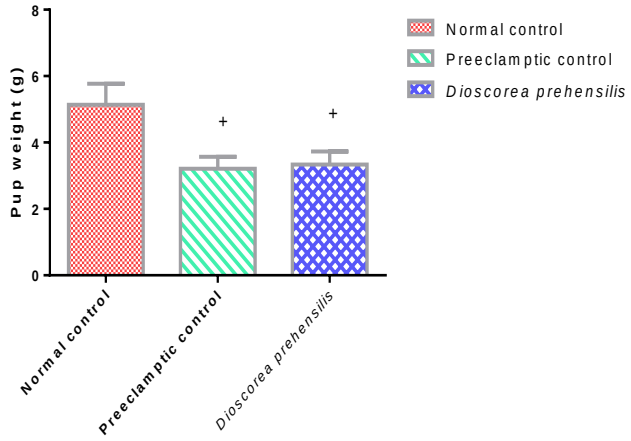


Figure 2: The pup weights of normal control, preeclamptic control and plant extract. Pup weights were obtained on day 20 of the experiment using a standard electronic balance, following the laparotomy and subsequent delivery. The data are expressed in grams (g) and presented as mean ±SD. Bars with (+) above are significantly different ($p < 0.05$) from the normal control.

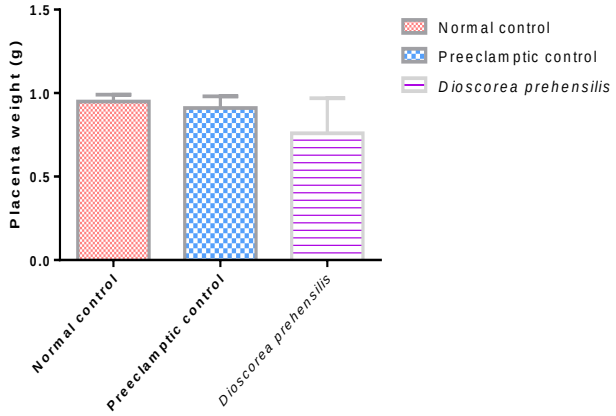


Figure 3: The placenta weights of normal control, preeclamptic control and plant extract. Placenta weights were obtained on day 20 of the experiment using a standard electronic balance, following the laparotomy, subsequent delivery and separation of the placenta. The data are expressed in grams (g) and presented as mean ± SD.

Albumin creatinine ratio

The albumin- creatinine ratios (ACR) from spot urine collected on day 19 gestation were compared among groups (Figure 4). The ACR in the normal group and the treated group were lower than that of preeclamptic group though not significantly.

Plasma nitric oxide levels

The plasma NO levels of all the groups were compared (Figure 5). There was no significant difference between the normal and preeclamptic control groups. The treated group had higher plasma NO levels when compared to both controls although; the difference did not reach statistical significance.

Litter size

The mean litter sizes of all the groups were compared (Figure 6). There was no significant difference among the normal control, preeclamptic group and the extract groups.

Histological analysis

The histological analysis of the placenta, liver, kidney and heart were carried out. The macroscopic examination of the placenta of the preeclampsia and treated groups showed peripheral and central infarcts indicative of an interruption in blood flow between the placenta and the baby, while that of the control group showed infarction only at the periphery of the tissue. Microscopic examination of the placenta of the normal control group showed regular villi and intervillus space while the preeclampsia group had excessive formation of syncytial knots, villus hypoplasia and intervillus congestion. The placentas of rats treated with DP also showed villus hypoplasia and intervillus congestion but not as severe as that obtained in the preeclampsia group (Figure 7).

Macroscopic and microscopic examination of the heart, liver and kidney of all the groups showed no remarkable differences between the normal, preeclamptic and the treated groups (Figure 8-10).

In this study, L-NAME induced preeclamptic model was employed to investigate the ability of the extract of DP to ameliorate the symptoms associated with preeclampsia. L-NAME is an analog of L-arginine, the substrate for nitric oxide synthase, NOS, and competes at the active site resulting in a reduction of the synthesis of NO.L-NAME induces a clinical picture similar to that of preeclampsia and when administered orally or intravenously, the effects are similar: hypertension, fetal growth retardation and proteinuria[20,21,22,23]. The elevation of blood pressure on the administration of L-NAME reflects its inhibitory action in reducing the production of NO. High blood pressure in preeclampsia is known to be due mainly to a reversal of the vasodilation characteristic of normal pregnancy, replaced by marked increases in peripheral vascular resistance[24]. Normally, the vasculature of normotensive gravidas manifests a decreased pressor responsiveness to several vasoactive peptides and amines, especially to angiotensin II. The vessels of women with preeclampsia however become hyper-responsive to these hormones, and in the case of angiotensin II, such changes may occur months before the appearance of overt disease[25]. Investigators have postulated that the vasoconstricting potential of pressor substances (e.g. angiotensin II and endothelin) is magnified in preeclampsia as a consequence of a decreased activity of NO synthase and decreased production of NO-dependent or -independent endothelium relaxing factor (EDRF).[26,27] The extract of DP improved the hypertension, causing a reduction in SBP. The lowering of the systolic blood pressure by this extract may be related therefore to the release of the potent vasodilator, NO. This is evident from the results of the nitric oxide assay which shows that the extract resulted in higher levels of NO compared to the normal and preeclamptic control, though not significantly.

A review of existing literature did not yield any information on the antihypertensive property of *Dioscorea prehensilis* but two different species of the same genus have been reported to have antihypertensive property. Amat*etal*[28] reported that *Dioscorea opposita* normalized hypertension in 2K1C hypertensive rats while Liu *etal*[29] reported the blood pressure lowering effect of *Dioscorea alata* in spontaneously hypertensive rats.

It has been established that L-NAME causes growth retardation in rats.[5,26] This was confirmed in the preeclamptic group which had significantly lower pup weights compared with the weights of the pups in the normal control group. The extract had no beneficial effect on

intrauterine growth retardation (IUGR) since it showed no significantly higher pup weight when compared to the preeclamptic control. Furthermore, the extract showed a significantly lower pup weight when compared with the normal control. This result is in agreement with some previous studies where the extract demonstrated antihypertensive effects but had no beneficial effect on IUGR.[5,30,31] Thus, a lowering of blood pressure does not seem to be associated with prevention of IUGR in this model.

The placenta weight of the preeclamptic control was lower than that of the normal control, though not significantly. The placenta plays a major role in the pathogenesis of preeclampsia.[32] This is evident in the fact that pathological examination of placentas from preeclamptic pregnancies generally reveals placental infarcts and sclerotic narrowing of arteries and arterioles, with characteristic diminished endovascular invasion by cyotrophoblasts and inadequate remodeling of the uterine spiral arterioles.[33] This is evident in the results of histological examination of the placenta obtained in this study. The normal control placentas showed regular villi and intervillus spaces while the preeclamptic group and the treated group showed some pathological changes including villus hypoplasia, intervillus congestion and excessive formation of syncytial knots, though the severity was reduced in the treated group. It is likely that the placental perfusion was somehow improved by the administration of the extracts.

The level of proteinuria in this study was assessed using ACR in spot urine. Measurement of protein excretion in a 24-hour urine collection has been the longstanding "gold standard" for the quantitative evaluation of proteinuria in pregnancy. However, 24-hour urine collection is time consuming, inconvenient, and not always reliable because of the difficulty in collecting the sample correctly.[34] Several studies have aimed at assessing the accuracy of a spot urine albumin/creatinine ratio in assessing proteinuria in preeclampsia in an attempt to ascertain its possibility of replacing the more cumbersome 24-hour urine collection and have found a close correlation between albumin/creatinine ratio and 24-hour albumin excretion and established that ACR may be a simple, convenient and accurate indication of proteinuria in preeclampsia.[34,35]

From the result of our study, the ACR in the normal control group was lower than that of the preeclamptic group confirming earlier reports that L-NAME elevates urinary protein excretion.[36]

The results of our study showed that when compared with the preeclamptic control, the extract lowered the ACR but not significantly. It has been documented that there is a gradual increase in proteinuria throughout pregnancy as a result of selective filtration and non-selective reabsorption in the proximal tubule.[37] This signifies a probable renal protective effect of this extract. The mechanism for proteinuria in preeclampsia is however not well understood.[38] The heart, liver and kidney were also investigated for pathological changes. No remarkable changes were observed in all of these organs in all the groups. Previous studies have established that in spite of a compromise in vascularity of all organs in preeclampsia, the liver, heart and brain are able to withstand this impoverishment much more easily and changes of necrosis usually appear in very severe form of the disease, like subjects dying of eclampsia.[39]

A previous study by our team showed that the methanol extract of DP possesses a significant dose dependent antiplatelet aggregatory property with an IC_{50} less than that obtained with the standard drug, aspirin. A similar inhibitory effect of a compound isolated from another specie of *Dioscorea* has also been reported.[40] Aberration in systemic prostaglandins, prostacyclin – thromboxane balance is known contributes to preeclampsia.[41] Prostacyclin antagonizes the vasoconstrictor, platelet-aggregating, and uterine-activating actions of thromboxane. It is believed that placental production of thromboxane is increased coincident with decreased production of prostacyclin in preeclampsia. During normal pregnancy, the placenta produces equivalent amounts of thromboxane and prostacyclin, so that their biologic actions on vascular tone, platelet aggregation, and uterine activity will be balanced.[42] In preeclamptic pregnancy, however, the placenta produces seven times more thromboxane than prostacyclin. This suggests that abnormal platelet activation occurs early in pregnancies destined to be complicated by preeclampsia and this activation may be involved in the pathogenesis of preeclampsia since its inhibition using low dose aspirin has been shown to modify the disease in high risk pregnancies.[43] Low dose aspirin seems to be the

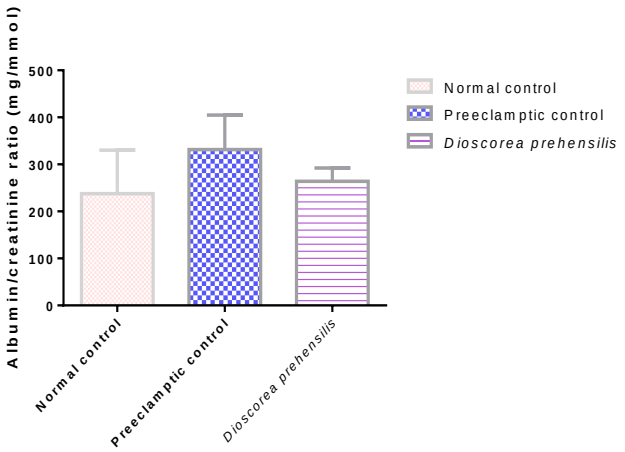


Figure 4: The albumin/creatinine concentration in spot urine samples of normal control, preeclamptic control and plant extract on day 19 gestation. The data are expressed in milligrams of albumin per mmol creatinine and presented as mean ± SD.

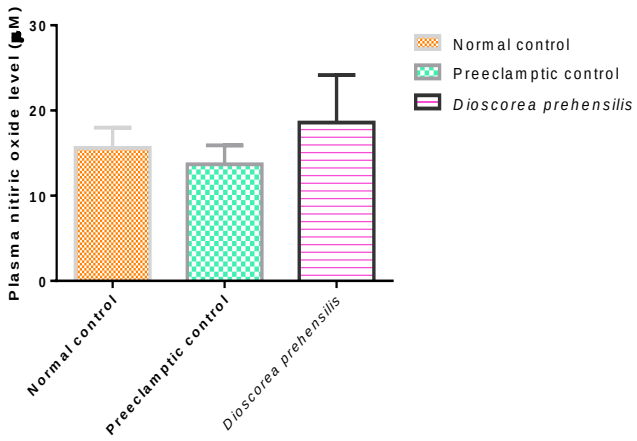


Figure 5: The plasma nitric oxide levels for normal control, preeclamptic control and plant extracts were determined by ELISA. The data are presented in micromoles (μM) and presented as means ± SD.

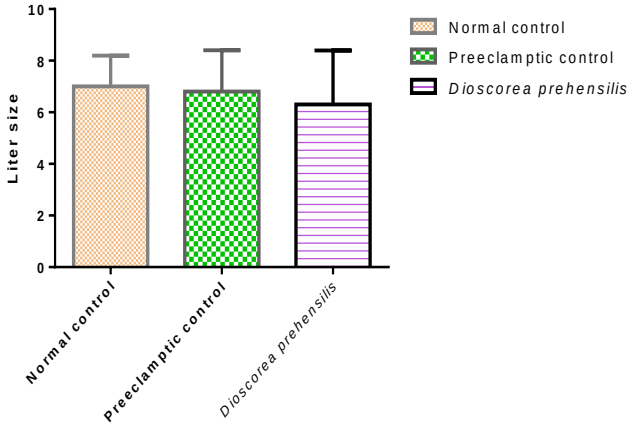
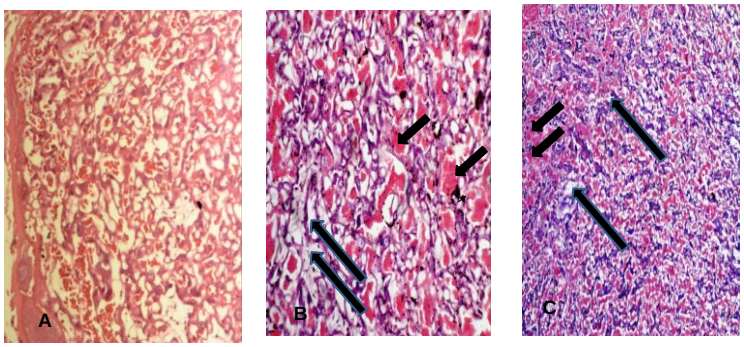
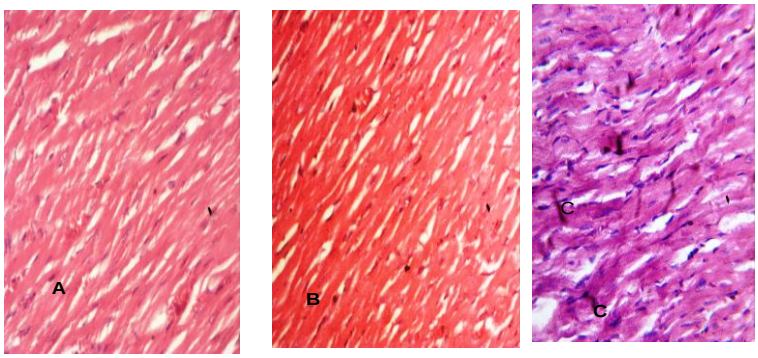


Figure 6: The liter size of live pups for normal control, preeclamptic control and plant extracts. The data are expressed as the average number of live pups for each group and presented as mean ± SD.



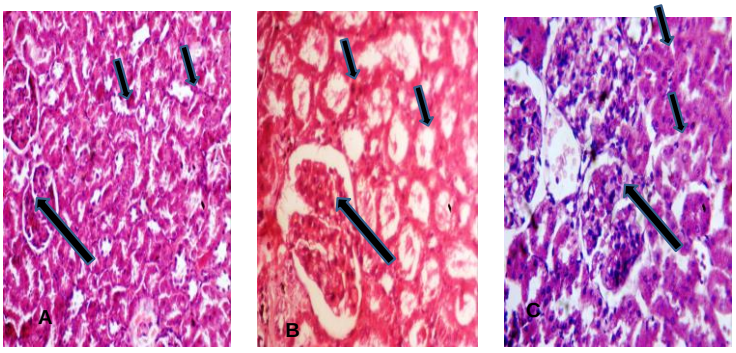
A. Normal, Placenta showing area of Regular villi and red blood cells within the intervillus space. **B. (Preeclampsia**), Hypoplastic Villi (Long arrow), intervillus congestion (Short arrow). **C: D.prehensilis,** Hypoplastic Villi (Long arrow), intervillus congestion (Short arrow), H & E X 100

Figure 7: Placenta histology showing hypoplastic villi and intervillus space congestion in the preeclamptic and treated group.



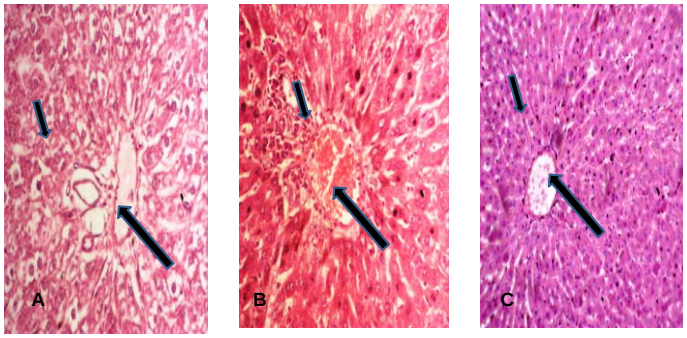
A= Normal, **B**= Preeclampsia, **C**= *D. prehensilis*. Sections showing regular cardiac myocytes. H& E X 100

Figure 8: Histological examination of the heart showing regular cardiac myocytes in all the groups.



A= Normal, **B**= Preeclampsia, **C**= *D. prehensilis*. Section show regular glomerulus (Long arrow), renal tubules (short arrow) and interstitium. H & E X 100

Figure 9: Histological examination of the kidney showing regular glomerulus and renal tubules in all groups.



A= Normal, **B**= Preeclampsia, **C**= *D. prehensilis*. Sections showing regular hepatocytes (Short arrow) and central vein (Long arrow). H& E X 100

Figure 10: Histological examination of the liver showing regular hepatocytes (short arrow) and central vein (long arrow) in all the groups.

most promising preventative therapy attempted currently and it is recommended in high-risk individuals. It inhibits platelets and thromboxane synthesis in order to inhibit platelet aggregation and vasoconstriction. It has been seen to reduce the incidence of preeclampsia by 9%.[44] The ability of this extract to improve the symptoms of preeclampsia may therefore among other factors, be related to its antiplatelet aggregatory property

Conclusion

It can be concluded from the results of this study that *Dioscorea prehensilis* contains bioactive compounds that are active against the components of preeclampsia and these may serve as a source of new drug entities for development in the management of preeclampsia.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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