SPERM CHARACTERIZATION, HORMONE ASSAY AND FERTILITY TEST IN MALE ALBINO WISTAR RATS TREATED WITH ETHANOL EXTRACT OF *Phyllanthus niruri*

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Abstract

Background: Failure to have a child is an unpleasant event in the lives of infertile people and is seen as the inability to conceive after at least one year of regular unprotected sex. About 30%-50% of the causes of infertility are related to male problems

Aim: The present study was aimed at evaluating the profertility effect of the n-hexane extract of whole plant part *Phyllanthus niruri* using male albino Wistar rats.

Materials and Methods: Daily oral doses of (10, 100 and 1000 mg/kg) the extract was administered to each animal in five different test groups for 28 consecutive days. Twenty-four hours after the last administration, all the animals were weighed and sacrificed. The blood was collected for reproductive hormonal assay such as luteinizing hormone (LH), follicle stimulating hormone (FSH), Estrogen and testosterone levels while the epididymis were isolated for sperm analysis and characterization respectively. Fertility test was carried out by monogamy mating method.

Results: The result revealed significant progressive increase ($p \leq 0.05$) in the body weight across the extract treated groups. Results obtained from the hormonal assay showed a dose dependent increase in the levels of testosterone but luteinizing hormones (LH), follicle stimulating hormone (FSH) and Estrogen significantly ($p \leq 0.05$) decreased dose dependently. Result obtained from the sperm analysis revealed significant increases in sperm motility and sperm count, while the sperm characterization showed normal sperm morphology in the extract treated animals when compared with the control (2 ml/kg). All the females mated to the treated males were pregnant and delivered twenty-one (21) days later.

Conclusion: These findings conclude that the N-hexane extract of the whole plant part of *Phyllanthus niruri* has pro-fertility activity in males and should be subjected to clinical trials.

Keywords: *Phyllanthus niruri*, albino rats, infertility, Profertility, Hormonal assay, Sperm analysis.

INTRODUCTION

Infertility, as a psychological crisis, imposes a lot of stress on infertile couples and in different ways threatens their mental health. About 30%-50% of the causes of infertility are related to male problems (Roozbeh *et al.*, 2016). Each day, the number of medical reports about the extent of infertility in the world increases, according to a systematic reviews in this regard, about 48.5 million couples around the world affected by this problem (Mascarenhas *et al.*, 2012).

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Infertility is a multi-parameter phenomenon with a wide range of factors that affects spermatogenesis and sperm quality (Imai *et al.,* 2001). Spermatogenesis is a process in which male sex cells are produced and the disorder in each of these stages can cause infertility (Bahrami *et al.,* 2014). Men fertility depends largely on the number, quality, motility, and morphology of the sperm, and the disruption of each of these factors leads to infertility in men (Roozbeh *et al.,* 2016).

Hormone Therapy, Surgical Procedures, Assisted Reproductive Technology (ART) that include in vitro fertilization (IVF), intra uterine insemination (IUI), zygote intrafallopian transfer (ZIFT), gamete intrafallopian transfer (GIFT), intracytoplasmic sperm injection (ICSI) and third-party fertilization (donation eggs, donation sperm, uterus, and donated embryos) are the various methods used to treat infertility (Baniaghil *et al.,* 2016). Regarding the problems that have been observed among couples and the high of medical interventions, people have turned to complementary medicine (Anbari and Ghanadi, 2015). Medicinal plants and their preparations have been used to treat several illnesses since the dawn of human history and are one of the foundations for healthcare worldwide (Oshomoh and Obaro, 2019).

Phyllanthus niruri is one of the species belonging to family *Euphorbiaceous* distributed throughout the tropical and subtropical regions of the world (Barros *et al.,* 2003). *Phyllanthus niruri* grows 30 - 40 cm in height, has small leaves and yellow flowers; the stem has green capsule, and blooms with flowers with 5 white sepals and apical acute anther (Cimanga *et al.,* 2004). The fruit has green capsules, and smooth and fruiting pedicels while seeds are longitudinally repose (Etta, 2008). It is found throughout the tropics and sub- tropics such as West Africa (including Nigeria and Ghana), Europe, Asia (including China, Pakistan, India and Malaysia Indian ocean), central and south America as medicinal plant for the treatment of various diseases (Jain *et al.,* 2008). The aim of this study is to evaluate and investigate the effect of the extract of *Phyllanthus niruri* on male fertility.

MATERIALS AND METHODS

Location of study

The study was carried out in the Phytomedicine laboratory of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State.

Laboratory Animals

Thirty healthy adult male and female albino Wistar rats (weighing between 150 – 180 g) were respectively obtained from the experimental animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. Animal were housed in the plastic cages in the animal house of Phytomedicine Unit, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State and feed with standard pellet diet and tap water at 25 ± 3^0C temperature, 50–60% humidity, and a 12 h light-dark cycle. Animals were acclimatized for one week before starting the experiment.

Plant Material Collection and Extraction

The whole plant part of *Phyllanthus niruri* was collected from the local garden within the premises of University of Benin during the raining season. The fresh plant collected was washed and air–dried for 14 days and final drying was done in oven at 40 ^{0} C until constant weight was recorded. The dried plant was grinded into fine powder. 1 kg of the grinded sample was thoroughly soaked and macerated using 2.5 L of 95% ethanol in a desiccator for 3 days. The mixture was then filtered using a cheese cloth concentrated in a rotary evaporator at 40ºC and then completely dried using an oven at 40ºC. The dried extract was stored in an airtight container. Appropriate doses of the extract were freshly prepared and administered orally to experimental rats daily (Obaro *et al*., 2021).

Experimental Design

The thirty (30) male albino rats were randomly categorised into five experimental groups of 6 animals each:

Group A: (control): administered feed and water only

Group B: 10 mg/kg/rat extract + feed and water

Group C: 100 mg/kg/rat extract + feed and water

Group D: 1000 mg/kg/rat extract + feed and water

Group E: Proviron 25 mg/kg + feed and water

Daily oral doses of the extract dissolved in distilled water were administered to each animal in the different groups using oro-gastric cannula, for a period of 21 consecutive days.

Body and Reproductive Organ Weight

Body weight of control and experimental animals were weighed at the beginning of the study and every seven (7) days respectively. 24 hours after the last doses of the extract, the rats were sacrificed by chloroform inhalation in a desiccator. Blood was collected for reproductive hormonal assay, testes and epididymis were dissected out and cleared of fat and connective tissue and weighed.

Hormonal Assay

After the animals were sacrificed 4 ml of blood was collected by cardiac puncture using 5 ml syringe and needles. The blood sample was placed in plane bottles and centrifuged at 2500 rpm for 10 minutes and serum used for testosterone (T), leutinizing hormone (LH) and follicle stimulating hormone (FSH) assay by the enzyme linked immunoassay technique.

Sperm Characterization

Sperm analysis was performed on samples derived from the caudal epididymis. The distal cauda epididymides were pulverized with scissors to release the epididymal content into a Petri dish containing 2 mL phosphate buffer (0.1 M, pH 7.4). The Epididymal sperm motility was assessed by calculating motile spermatozoa per unit area and was expressed as percentage motility. Epididymal sperm counts were made using the haemocytometer and were expressed as million/ml of suspension. The sperm viability was also determined using Eosin/Nigrosin stain as earlier described (Raji *et al*, 2003).

Fertility Test

Male rats treated for twenty-one (21) days were introduced to females with confirmed oestrus in the ratio 1:1 for a period of seven days and the ratio of pregnant to un-pregnant females was recorded.

Histological Processing

This was done as essentially as described by Osazuwa *et al.* (2020). The organs were cut in slabs of about 0.5 cm thick transversely and fixed in Bouin's fluid for a day after which it was transferred to 70% alcohol for dehydration. The tissues were passed through 90% alcohol and chloroform for different durations before they were transferred into two changes of molten paraffin wax for 20 minutes each in an oven at 57^0 C. Serial sections were cut using rotary microtome at 5 microns. Slides were prepared from these tissues. The slides were de-waxed and passed through absolute alcohol (2 Changes); 70 % alcohol and then to water for 5 minutes. The slides were then stained with haematoxylin.

Statistical Analysis

Data are expressed as mean ± SEM and analysed using the student's test and ANOVA where necessary. $P \leq 0.05$ was accepted as significant.

RESULTS

Effect of *P.niruri* on Body weight index of male albino rats

All tested doses and the positive-control (Proviron) were noticed to be significantly different ($P \leq 0.01$) from each other on the respective days (1, 7, 14 and 21) of testing, ($P \leq 0.001$) were significant differently from each other on the respective days (1, 7, 14 and 21) and ($P \leq 0.0001$) were significant differently from each other on the respective days (1, 7, 14 and 21) (Table 1).

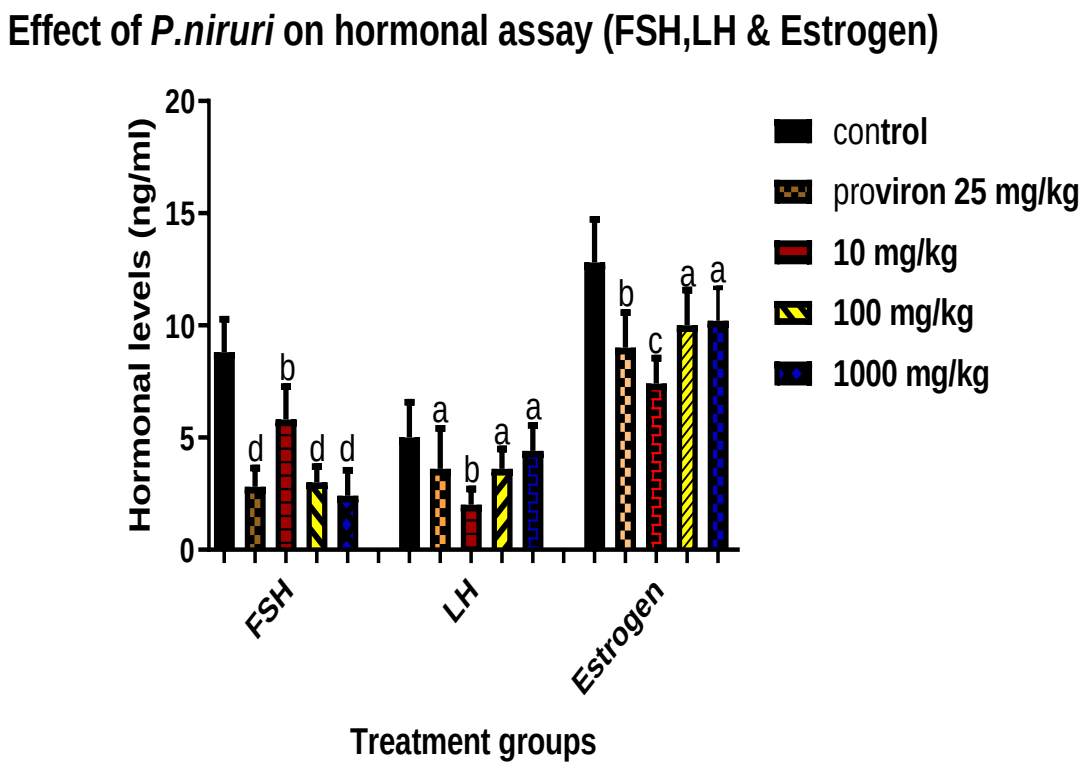
Table 1: Effect of *P.niruri* on Body weight index of male albino rats

Treatment groups (mg/kg)	**Initial weight**	**Weight at day 7**	**Weight at day 14**	**Weight at day 21**
Control	152 ±1.28	156 ±1.18	159 ±1.07	161 ±0.98
10	155 ± 1.29	164 ± 1.4	173 ± 1.20[b]	179 ± 1.07[b]
100	153 ± 1.24	160 ± 1.43	165 ± 0.55	173 ± 0.58[b]
1000	156± 1.31	178± 1.37[c]	184 ± 1.47[d]	188 ± 1.52[d]
Proviron 25	154± 1.09	179± 1.02[c]	184± 4.38[d]	189±13.44[d]

Results are expressed as mean ±SEM, n=6 for all treatment group. [b] =p≤0.01, [c] =p≤0.001, [d] =p≤0.0001

Effect of *P.niruri* on Sex hormones (FSH, LH and Estrogen)

A significant decrease in the levels of FSH at the tested dose was observed from 9 to (3, 6, 3 and 2.5) (Figure 2). As shown in figure 2, a significant decrease in the level of LH at the tested dose was observed from 5 to (4, 2, 4 and 5) and a significant decrease in the level of Estrogen at the tested dose was observed from 14 to (9, 8 and 9.5).

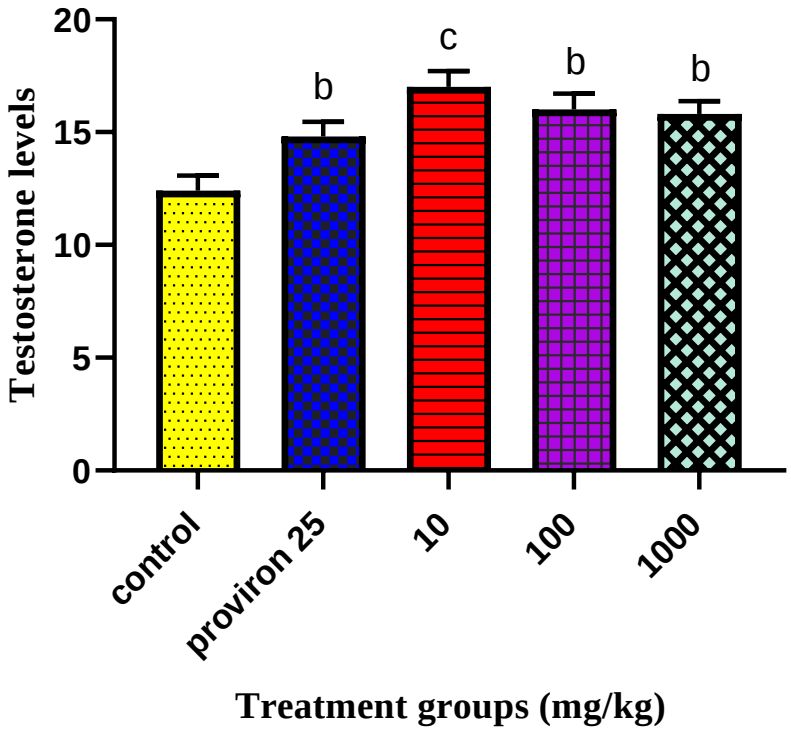


Results are expressed as mean ±SEM, n=6 for all treatment group. [a] =*p≤0.05*, [b] =*p≤0.01*, [c] =*p≤0.001*, [d] =*p≤0.0001*

Figure 3: Effect of *P.niruri* on hormonal assay (FSH, LH and Estrogen).

Effect of *P.niruri* on hormonal assay (Testosterone)

The effect of ethanol extract of *P.niruri* on Testosterone level in the male rats after 21 days of treatment are displaced on (Figure 4). All test dose groups were found to be significant at 16, 15.5 and 15 respectively.



Results are expressed as mean ±SEM, n=6 for all treatment group. [b] *=p≤0.01*, [c] *=p≤0.001*.
Figure 4: Effect of *P.niruri* on hormonal assay (Testosterone)**.**

Effect of *P.niruri* on sperm count, sperm viability, progressive motility, non-progressive motility, immotile, normal and abnormal.

Sperm characteristics after 21 days of treatment review increases in total sperm count from 346±10.30 to 422.0±12.41 (*P≤0.01*), 450.0±18.44 *(P≤0.001*) and 486.0±10.30 (*P≤0.0001*). Progressive Motility after 21 days of treatment review increases from 90.2±1.59 to 92.20±0.86, 92.8±0.58, and 94.80±1.20 (*P≤0.05*). Non-Progressive Motility after 21 days of treatment review decreases from 10.0±0.71 to 7.80±0.49 (*P≤0.05*), 8.2±0.67 and 5.20±0.37 (P<0.0001). Abnormal after 21 days of treatment review decreases from 5.80±1.11 to 3.00±0.71, 3.00±0.71 and 2.80±0.58 (*P≤0.05*).

Table 2: Effect of *P.niruri* on sperm count, sperm viability, progressive motility, non-progressive motility, immotile, normal and abnormal.

Treatment groups (mg/kg)	Total sperm cell count 10^6cells/mm^3	Progressive Motility	Non Progressive Motility	Immotile	Normal	Abnormal
Control	346.0±10.30	90.20±1.59	10.0±0.71	3.0±0.8	95.40±0.51	5.80±1.11
10	422.0±12.41[a]	92.20±0.86	7.80±0.49[a]	3.20±0.73	96.40±0.68	3.00±0.71
100	450.0±18.44[c]	92.80±0.58	8.20±0.67	2.40±0.68	96.20±0.86	3.00±0.71
1000	486.0±10.30[d]	94.80±1.20[a]	5.20±0.37[d]	2.40±0.75	95.20±1.39	2.80±0.58[a]
Proviron (25)	400.0± 9.45[a]	90.70± 2.10	10.20±0.39	2.95±0.67	95.67±2.76	6.00±0.64

Results are expressed as mean ±SEM, n=6 for all treatment group. [a] *=p≤0.05*, [c] *=p≤0.001*, [d] *=p≤0.0001*

Table 3: Fertility Effect of male rats treated with ethanol extract of the whole plant of *P.niruri*

Treatment group (mg/kg)	No. of exposed male:female	Positive mating (%)	Fertility (%)
Control	6:6	100	100
10	6:6	100	100
100	6:6	100	100
1000	6:6	100	100
Proviron (25)	6:6	100	100

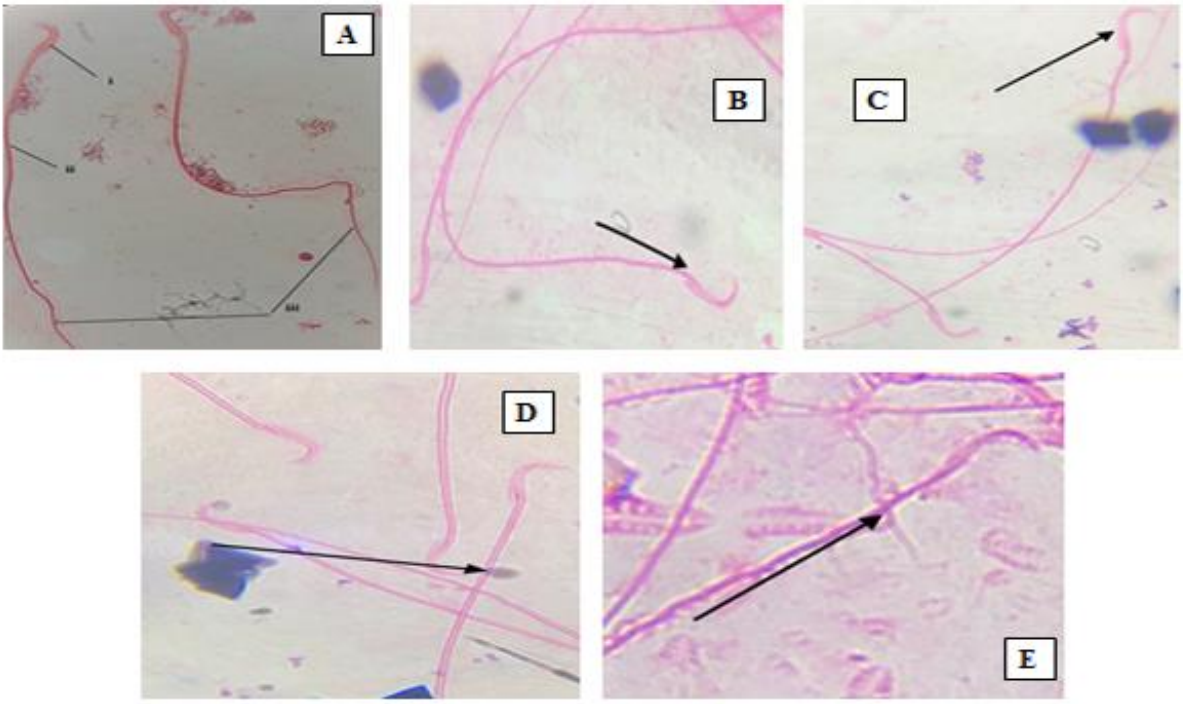


Plate A, B, C, D and E: Micrograph of sperm cells from adult male Wistar rats treated with rats treated with ethanol extract of the whole plant of *P. niruri* (10. 100 and 1000 mg/kg) for 28 days.

Key:

A= Control- Normal Sperm cell morphology, with well-formed head, body and tail, which is labelled as i, ii and iii

B= Ethanol extract of the whole plant of *P.niruri* (10 mg/kg)- Normal Sperm cell morphology, with well-formed head, body and tail, which is labelled as long arrow.

C= Ethanol extract of the whole plant of *P.niruri* (100 mg/kg)- Normal Sperm cell morphology, with well-formed head, body and tail, which is labelled as long arrow.

D= Ethanol extract of the whole plant of *P.niruri* (1000 mg/kg)- Normal Sperm cell morphology, with well-formed head, body and tail, which is labelled as long arrow.

E= Proviron (25 mg/kg)- Normal Sperm cell morphology, with well-formed head, body and tail, which is labelled as long arrow.

DISCUSSION

The rise in body weight in test rats suggests that the extract did not interfere with the body's fat depots, possibly via increased metabolism and increased storage of fat and carbohydrate in the body. A combination of these processes has been implicated in the increasing of body weight gains (Ijioma *et al.,* 2018). The extract in the gastrointestinal tract may have exerted excitatory effects on the gastrointestinal smooth muscles leading to increased transit of food substances and increased appetite for additional food which ultimately may increase glucose available for body use and thereby affecting body weight (Cummings and Overduin, 2007).

The increase in fertility potentials reported after the treatment of male rats with *P.niruri* ethanol extract can be attributed to enhancement in sperm motility and viability (Nwanjo *et al.,* 2007). The findings of the present study showed that the ethanol extract of *P.niruri* could significantly increase the fertility potential of male rats. The fact that there was significant effect on the body weight on treated animals rule out the possibility of a systemic toxicity at the doses treated since there was no behavioural alterations observed within the treated group. The treated groups showed normal agility compared to control. Furthermore the significant increase in the weight ($p \leq 0.01$) of the treated groups indicates that the extract may have toxic effect on this organ (Simons *et al.,* 1995).

Similarly, the increase in sperm qualities points to increase in the circulating androgen level. Any chemical agent that can affect reproductive activity will as well affect the quality and quantity of the sperm. The sperm counts, motility and viability of the treated samples were significantly increased in treated groups when compared to the control which indicates that at these concentrations the extract may not be toxic. These observations show that the ethanol extract of *P. niruri* may have profertility effects in albino rats at doses 10 mg/kg, 100 mg/kg, 1000 mg/kg. The sperm characterization revealed no toxic effect from the normal morphology of the treated rats. There was 100 % record in all the treated groups as all the female rats mated were confirmed to be pregnant and gave birth after 21 days of mating. But since there has been no such documentation of the plant with respect to humans research should be directed towards this area to assess the effect of the extract on man.

The findings of the present study also showed that the ethanol extract of *P. niruri* significantly altered the serum leuteinising hormone, follicle stimulating hormone and testosterone levels in the test rats. The follicle stimulating hormone released from the anterior pituitary gland decreased significantly, as observed in this study. The significant ($P \leq 0.01$ and 0.001) increase in testosterone, especially in the higher test doses, can be attributed to the decrease in leuteinising hormone and follicle stimulating hormone. This finding agrees with that of Obianime and Uche, (2008) who reported that the extract caused an increase in the level of testosterone in Guinea pigs.

Conclusion

The result of this study has shown that the ethanol extract of the whole part of *Phyllanthus niruri* significantly altered the levels of reproductive hormones and sperm characteristics of the male albino rats, and fertility in all treated male rats. Therefore, local care givers and traditional medical practitioners can subject it to clinical trials in infertile males.

Author Contributions

Dr. Obaro P. O. and Dr. (Mrs) Obaro-Onezeyi O. E. conceived and designed the research; Dr. Obaro P.O. performed the experiments; Dr. (Mrs) Obaro-Onezeyi O. E. analysed the data; Dr. Obaro Peter and Dr. (Mrs) Obaro-Onezeyi O. E. contributed reagents/materials/analysis tools; Dr. Obaro P.O. wrote the manuscript. Dr. (Mrs) Obaro-Onezeyi O. E. read through the manuscript before publication.

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Contending interests
Authors declare that they have no contending interests.

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