British Journal of Applied Science & Technology
4(15): 2194-2205, 2014

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Estrogenic and Hypocholesterolemic Effects of Aqueous Extract of *Nicotiana tabacum* (Tobacco) on the Micro-Anatomical Architecture of the Testis in Male Albino Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author JOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors AAN, SIO, AOO, and FO managed the analyses of the study and participated during the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 15th February 2014
Accepted 20th March 2014
Published 31st March 2014

ABSTRACT

Aim: The aim of this study was to investigate the effect of aqueous extract of *Nicotiana tabacum* on the testicular function and micro-architectural integrity in male Albino Wistar rats in relation to the serum concentrations of estradiol, cholesterol, testis and body weights.
Study Design: The experiment was carried out on Albino Wistar Rats.
Methodology: A total of 18 male Albino Wistar rats aged 8 to 10 weeks and weighing 140 to 220 g was used. Aqueous extract of *N. tabacum* at the doses of 20 and 30 mg/kg body weight were orally administered to groups B and C, respectively while 0.5 ml of distilled water was administered to group A (Control) for 28days. Serum concentration of estradiol (E2) and total cholesterol were estimated using the microplate enzyme

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immunoassay and enzymatic end point methods, respectively. The SPSS software (version 20) was used for the statistical analysis and the result expressed in mean ± SEM.
Result: The result showed significant increase in serum estradiol concentration (from 2.50±0.43 pg/ml in the control group to 9.33±1.87 pg/ml in the treated groups), significant decrease in serum total cholesterol concentration (from 1.92±0.04 mmol/l in the control group to 1.22±0.08 mmol/l in the treated groups) and body weight ($p \leq 0.05$), and insignificant increase in the weight of the testes ($p \geq 0.05$). Testicular microscopy showed decreased spermatogenesis, atrophic interstitial space and moderate hypertrophy of the seminiferous tubules.
Conclusion: Therefore, *N. tabacum* could be considered a potential endocrine disruptor which can affect the micro-anatomical architecture and function of the testis.

Keywords: Testicular microscopy; estradiol; total cholesterol; Nicotiana tabacum; albino wistar rats; body weight; testicular weight; spermatogenesis; micro-anatomy.

1. INTRODUCTION

Tobacco is a name for any plant of the genus Nicotiana of the Solanaceae family (nightshade family) and the products manufactured from its leaf include cigars, cigarettes, snuff and chewing tobacco [1]. The smokeless tobacco has different nature names, these are: Ntsu in South Africa, Toombak in Sudan, Shammah in South Arabia, plug chew in the United States and in Nigeria, the common names are Anwuru in Igbo, Taba in Yoruba and Hausa languages [2].

Chewing tobacco has been known to cause cancer, particularly of the mouth and throat [3], however some health scientists suggest that smokeless tobacco should be used in smoking cessation programmes and have made implicit or explicit claims that its use would partly reduce the exposure of smokers to carcinogens and the risk for cancer.

Tobacco (smoked or smokeless) have been suggested to contain other phytochemical constituents apart from nicotine such as potent tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), 4-methyl-nitrosamino)-4-(3-pyridyl)-butanal (NNA), and N-nitrosonornicotine, heavy metals (Cadmium (Cd), mercury (Hg) etc) and 23 polycyclic aromatic hydrocarbons which has been implicated with tobacco associated cancers and diseases [4-10]. Smokeless tobacco comes in two different forms, which are 'Tobacco snuff' and 'Chewing tobacco' [11]. Tobacco snuff is the powdered form blended with potash as the main additive in Nigeria [12] and has been recommended as a substitution for cigarette since it is devoid of hazardous elements such as tar and carbon monoxide [13]. For this reason, many people believe that using smokeless tobacco is safer than smoking it. This however, is not true because smokeless tobacco can induce addiction to nicotine and leukoplakia [14]. The male reproductive system is known to be highly sensitive to many chemicals and drugs which have been found to pose adverse effects on male reproductive capacity under certain conditions [15].

Although traditionally, estradiol is considered the female sex hormone, but through a number of experimental models, it has been shown that estradiol plays a vital role in normal sperm cell development and function [16-18]. Estradiol is produced in the testes from aromatized testosterone. Progressive disruption of spermatogenesis and infertility has been observed among aromatase knockout mice [19]. A direct role of estradiol as a germ cell survival factor

has been demonstrated in the human testis in vitro, where estradiol was shown to inhibit testicular apoptosis much more effectively (100- to 1000-fold) than testosterone [20]. Estradiol has also been shown to induce spermatogenesis in gonadotropin-deficient mice [21-23].

Some studies have provided novel insight into the molecular basis for spermiation failure and apoptosis caused by 17beta-estradiol and also offer new mechanisms by which adult exposure to environmental estrogens can affect spermatogenesis and fertility [24]. Studies on boys delivered by mothers treated with diethylstilbestrol, a very potent estrogen agonist from 1950 to 1970, have reported alterations in sperm quality and higher incidence of genital malformations, cryptorchidism, and testicular cancer compared to the control population [25-26]. Estrogenic drugs administered by injections, oral gavage, or via drinking water have been claimed to result in varied effects such as decreased Sertoli cell number, Leydig cell hyperplasia, decreased sperm count and testicular weight [27-28]. There is growing evidence suggesting a decline in fertility in humans and also an increased incidence of testicular cancer after exposure to environmental oestrogen and endocrine disruptors [29]. Cholesterol functions as a metabolic precursor for the biosynthesis of bile acids, and steroid hormones which include male and female sex steroids (androgens and oestrogens). The aim of this study was to investigate the estrogenic and hypocholesterolemic effect of aqueous extract of *N. tabacum* on the testis in male Albino Wistar rats.

2. MATERIALS AND METHODS

Test sample: Leaves of *Nicotiana tabacum* were collected and identified by a botanist at the Department of Botany, Nnamdi Azikiwe University Awka, Anambra State.

2.1 Sample Extraction

100 g of the plant material was extracted by maceration in 1000 ml of distilled water with intermittent agitation (8 hrs days) for 3 days using mechanical shake. Afterwards the mixture was filtered using filter paper (whatman) and the filtrate concentrate by rotary evaporation to dryness and yield 23.5g (23.5) of solid residue was obtained. The residue in form of pest dark brown product was transferred to an airtight bottle and stored in the refrigerator at -4°C until use [30].

2.2 Experimental Design

A total of 18 male Albino Wistar rats aged 8 to 10 weeks and weighing 140 to 220g was used for this study. The animals were examined by a veterinarian for overall health condition and allowed to acclimatize for two weeks at the Animal House of the College of Health Sciences, Nnamdi Azikiwe University, Nnewi campus. The animals were randomly divided into three groups, containing 6 male Albino rats each. The experimental design consisted of three groups designated Group A (control), B and C. Group B and C were orally administered sub-lethal doses of 20 and 30 mg/kg, of the *N. tabacum* extract respectively with due consideration to their body weight via orogastric tube. The doses were mixed in 0.5 ml of distilled water. Group A (control) were orally administered equal volume of distilled water as well (0.5 ml) without *N. tabacum*.

The experiment was conducted in accordance with the Guidelines of the U.S. National Institute of Health (NIH) on the care and use of laboratory animals. The animals were kept

under standard and good laboratory conditions (which include: 12 hour light and 12 hour darkness, temperature of 30°C±4.5°C, humidity and ventilation). Prior to exposure, the animals were starved of solid food overnight and their body weights taken before (and after) the experiment to check for weight loss or gain which is associated with toxicity. The animals were fed vital finisher mesh (from Vital Feed Ltd, Jos, Plateau State, Nigeria) and water *ad libitum* for 28 days.

2.3 Animal Sacrifice, Sample Collection and Histology

On the 29th day morning, animals were anaesthetized using cotton wool damped in chloroform with due consideration of their body weights. Blood (2 ml) was collected from each animal via the retro-orbital sinus with 70 µl heparinized capillary tube, dispensed into plain sample bottle [31] and centrifuged (Rotofix 32®-Hettich) at 3000 g for 10 min; the serum was collected and kept at –20°C until analysis. Animals were dissected and both testes were excised, trimmed of all fat, blotted dry to remove traces of blood and fluid, and weighed using an electronic weighing balance (210/0.1 mg digital balance ESJ-210-4). The excised testes were grossed, fixed in 10% formal saline (a fixative) and processed through paraffin wax to obtain the tissue blocks. Testes slices of 3 µm thicknesses were obtained from the tissue blocks via microtomy, stained using Haematoxylin and Eosin (H&E) staining Technique [32] and photomicrograph of the stained tissue sections were taken (by a microscope which had a camera attached to it) for documentation. The processing of the testes was carried out at Federal Medical centre Owerri, Imo State, Nigeria.

2.4 Biochemical and Hormonal Assay

Serum concentration of estradiol was estimated using the microplate enzyme immunoassay [33], (using a kit from Monobind Inc., USA.) and total cholesterol was estimated by the enzymatic end point method (using akit from Randox Laboratories, United Kingdom). These biochemical and hormonal assay were carried out using spectrophotometer and ELISA machine, MR 96 USA, respectively. The latter was carried out using the facilities of Reene Laboratories Onitsha, Anambra State.

2.5 Statistical Analyses

Mean values (±SEM) of serum concentrations of estradiol, total cholesterol including the body and testes weights were taken for analysis. The data was tested for homogeneity of variance and significantly different results were established by one-way ANOVA using the SPSS software application (version 20). The multiple comparisons were made using the Post hoc test. The accepted level of significance was set at $p \leq 0.05$. The Pearson's correlation was also made to correlate the serum concentrations of estradiol and total cholesterol, the weight of the animals before and after the experiment, and the accepted level of significance set at 0.01. The Paired sample T-test was used to compare the body weight of the animals before and after the experiment and the significance set at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Behavioural Effect

After five days of administration, the animals in the treated groups B and C lost their appetite. Considering the fact that all the groups received approximately the same amount of food, it

was observed that the treated groups had reduction in food consumption (having ruminants) when compared with the control group. They also became less active when compared with the control group. Soon after the 9th day, they regained their appetite, though not completely for the duration of the experiment.

3.2 Effect on Body Weight

The mean ± SEM of the body weight of the animals in group A, B and C before the experiment were 148.33±4.59, 175.83±3.27 and 209.17±6.64 g respectively, but these values changed to 172.50± 4.03, 181.67±7.15 and 204.17±9.87 g respectively at the end of the experiment (Fig. 1). The paired sample T-test revealed a decrease in body weight of the animals in the treated groups when they were compared with the control group ($p \geq 0.05$), but a significance decrease was only observed in group C ($p \leq 0.05$) when the Pearson's correlation was carried out (Table 3).

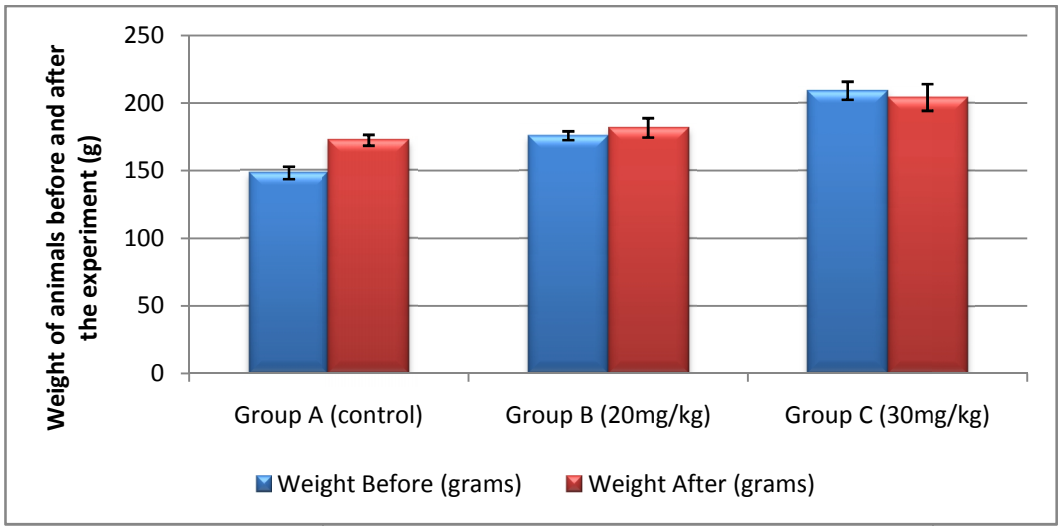

Fig. 1. A graphical comparison of the mean (±SEM) weight of animals in the control and treated groups before and after the experiment (One-way ANOVA)

3.3 Histopathological Findings and Legends

The table above shows a significant increase serum estradiol and significant decrease in the serum total cholesterol concentrations when the treated groups is compared with the control group ($p \leq 0.05$). More so, the table shows insignificant increase in the weight of the testes when that of the control group was compared with the testes of the treated groups ($p \geq 0.05$). One-Way ANOVA and Post Hoc Test.

The table above shows that there is a significant increase in the weight of the animals in the control group when their weight before the experiment was compared with their weight after the experiment ($p \leq 0.05$). The decrease in weight observed when the weight of the treated groups before treatment was paired with their weight after the treatment showed that N. tabacum has a dose dependent effect on body weight. Paired sample T-test.

The table above shows a strong correlation between the weight (of the animals in the treated groups) before and after the experiment in relation to the dosage of the *Nicotiana tabacum* administered, despite the fact that Table 2 showed insignificant decrease in the weight of the animals. However, it was able to show that the effect of the extract (at 30 mg/kg) was significant in the group C ($p \leq 0.05$) Pearson's Correlation.

The table above shows a significant and strong inverse relationship (correlation) between serum cholesterol and estradiol ($p \leq 0.05$). This suggests that a decrease in serum concentration of cholesterol is associated with an increase in estradiol concentration.

Cigarette (Tobacco) smoking has been shown to have anti-estrogenic effects in women [34]. Thus, the result of this study highlights the potential effect of aqueous extract of *N. tabacum* on serum concentrations of estradiol and cholesterol in relation to the cytoarchitecture of the testis and overall body weight.

Nicotine has been shown to have adverse effects on fertility potentials of female albino rats by reducing the weight and disorganizing the histology of some vital visceral and reproductive organs [35]. Oral administration of nicotine have been associated with testicular degeneration, disorganization of the cytoarchitecture and decreased serum testosterone levels [36]. The dose dependent increased of estradiol (Table 1) observed in the treated groups may be an indication of increased aromatisation of testosterone. This could be linked to previous findings [36] which reported a dose dependent decrease in testosterone concentration following administration nicotine (at the doses of 0.5 mg/kg and 1.0 mg/kg body weight) to male Sprague-Dawley rats for 30days.

Table 1. Mean±SEM of serum concentration of estradiol, cholesterol and testes weight in the control and treated groups

Parameters	Groups			F-value	P-value
	Group A (control)	Group B (20 mg/kg)	Group C (30 mg/kg)		
Estradiol (pg/ml)	2.50 ±0.43	4.83 ±0.60 (0.176)	9.33 ±1.87 (0.001)	8.925	.003
Cholesterol (mmol/l)	1.92 ±0.04	1.47 ±0.15 (0.007)	1.22 ±0.08 (0.000)	12.177	.001
Weight of testes (grams)	2.75 ±0.09	3.22 ±0.07 (0.114)	2.97 ±0.29 (0.424)	1.712	.258

P –value is significant at p≤0.05. n=6

The histopathological changes observed in the photomicrographs from the treated groups (Figs. 3 and 4) when compared with that of the control group (Fig. 2) are in accordance with the reports of prior studies [36]. They are also in agreement with earlier findings [30], which reported a significant decrease in sperm concentration and percentage motility following administration aqueous extract of *N. tabacum* to Albino Wistar rats (in the dosage of 20 mg/kg and 30 mg/kg body weight) for 21 days. The possible mechanism of the observed toxicity could be by stimulation of hormone production from the adrenal cortex, causing a negative feedback on gonadotropin-releasing hormone in the pituitary gland to suppress spermatogenesis.

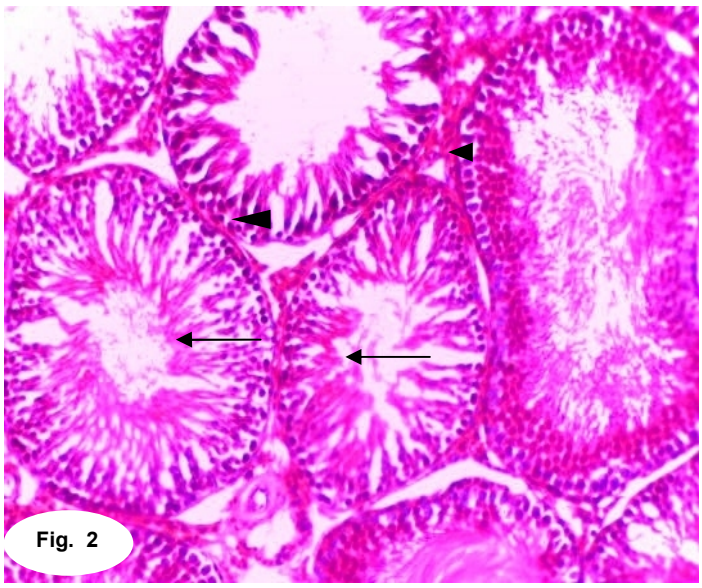

Fig. 2-Group A (control): a section of the testis featuring the seminiferous tubules enclosing the spermatozoa (marked by arrows), the interstitial space (marked by arrow head at the top) and basal lamina (marked by a slightly larger second arrow head below). Stained by Haematoxylin and Eosin technique. X200

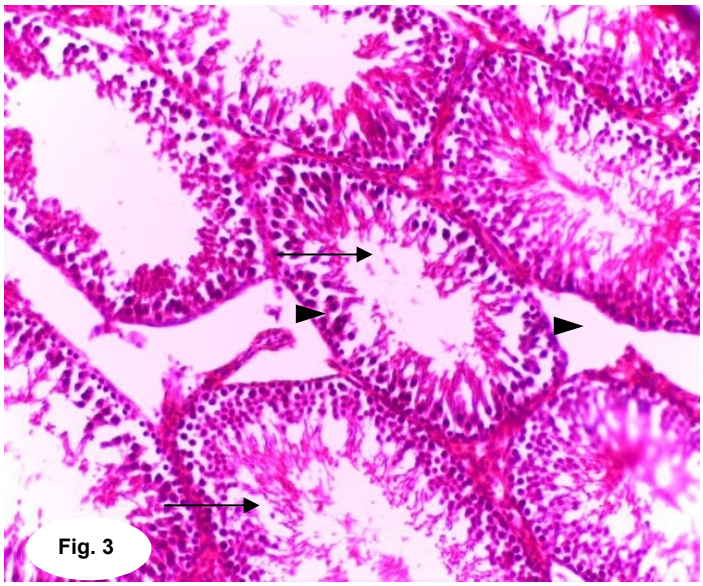

Fig. 3-Group B (20 mg/kg): a section of the testis with decreased spermatozoa in the seminiferous tubules (arrows). The section also shows atrophic interstitial space (housing the Leydig cells and Sertoli cells) and basal lamina (arrow heads). Stained by Haematoxylin and Eosin technique. X200

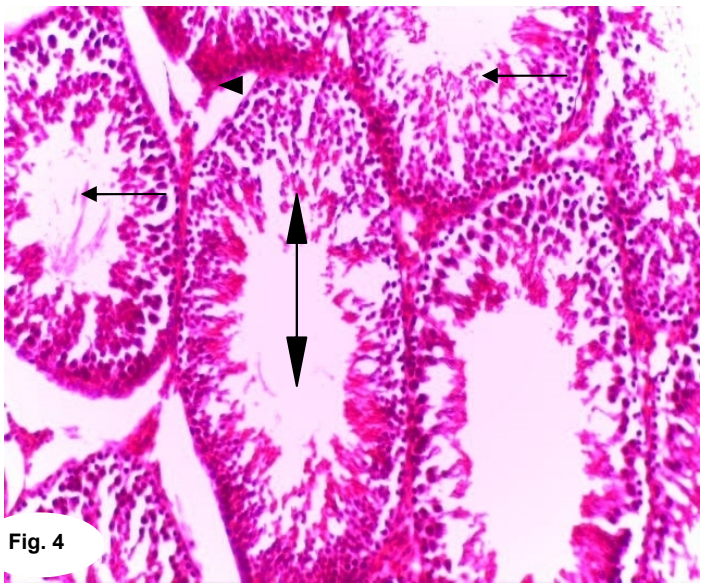

Fig. 4-Group C (30 mg/kg): a section of the testis with decreased spermatogenic activity, atrophic interstitial space, moderate hypertrophy of the seminiferous tubules (marked by a double headed arrow at the center). Stained by Haematoxylin and Eosin technique. X200

However, the insignificant increase in weight of the testis observed in the treated groups (B and C) of this study (Table 1) is not in consonance with previous findings [36], which reported a significant decrease in testes weight of the treated groups when compared with the control groups. The increase in testicular weight observed in this study could be linked to the moderate hypertrophy of the seminiferous tubules which was more pronounced in animals that received 30 mg/kg body weight of the extract.

The paired sample comparison (Table 2) revealed a decrease in body weight of the animals in the treated groups when they were compared with the control group, but a significance decrease was only observed in group C (Table 3). This suggests that the *Nicotiana tabacum* has a dose dependent effect on the weight of the treated animals. The loss of appetite observed in the treated groups during the experiment could have contributed to the decrease in body weight after the experiment (Fig. 1).

Table 2. Paired sample T-test of the weight of the animals before and after the experiment

Body Weight of animals(g)		Mean ± SEM	t	P-value
Pair 1	Group A before - Group A after	-24.17 ±5.07	-4.768	.01
Pair 2	Group B before - Group B after	-5.83 ±4.90	-1.190	.29
Pair 3	Group C before - Group C after	5.00 ±5.63	0.889	.42

P –value is significant at p≤0.05. n=6 Keys: Group A (Control: 00 mg/kg aqueous extract of Nicotiana tabacum) Group B (20 mg/kg aqueous extract of N. tabacum) Group C (30 mg/kg aqueous extract of N. tabacum)

Cholesterol functions as a major metabolic precursor for the biosynthesis of bile acids, and steroid hormones which include male and female sex steroids (androgens and oestrogens) and adrenal steroids (aldosterones and corticosterone) and liver, ovaries, testes and adrenal glands are these the main producers of these hormones using cholesterol as the main precursor [37]. The serum cholesterol concentration (Table 1) was found to have a strong inverse relationship with estradiol concentration (Table 4). In essence, this suggests that the decrease in serum cholesterol concentration must have led to the observed increase in estradiol concentration. Hence, it could be adduced that cholesterol was metabolised (leading to its decrease) to testosterone which was in turn aromatised to estradiol (resulting in its increase).

Table 3. Paired Sample Correlation of the weight of the animals before and after the experiment

Weight of animals		Correlation	*P*-value
Pair 1	Group A before & Group A after	0.315	.543
Pair 2	Group B before & Group B after	0.808	.052
Pair 3	Group C before & Group C after	0.838	.037

Correlation is significant at 0.05 (2-tailed). n=6 Keys: Group A (Control: 00 mg/kg aqueous extract of Nicotiana tabacum) Group B (20 mg/kg aqueous extract of N. tabacum) Group C (30 mg/kg aqueous extract of N. tabacum)

Table 4. Correlation between serum concentration of Cholesterol and Estradiol between the groups

Variables	r-value	*p*-value	Remark
Cholesterol correlated with estradiol in males	-0.582*	0.011	Negative correlation

**. Correlation is significant at the 0.05 level (2-tailed). n=6 Keys: Group A (Control: 00 mg/kg aqueous extract of Nicotiana tabacum) Group B (20 mg/kg aqueous extract of N. tabacum) Group C (30 mg/kg aqueous extract of N. tabacum)*

4. CONCLUSION

Thus, this study suggests that smokeless *Nicotiana tabacum* might possess estrogenic and hypocholesterolemic properties, and has the potential to affect the micro-anatomical architecture and function of the testis.

ETHICAL APPROVAL

All authors hereby declare that the experiment was conducted in accordance with the guidelines of the U.S. National Institute of Health (NIH) on the care and use of laboratory animals and also followed the protocol for handling of laboratory animals as stipulated by the ethics committee, Faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus.

COMPETING INTERESTS

Authors declare that there are no competing interests.

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