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Reproductive Functions in Male Rats Treated With Methanolic Extract of *Alstonia Boonei* Stem Bark

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Abstract

The effects of methanolic extract of *Alstonia boonei* stem bark was studied on sperm characteristics, fertility, body and organ weights of adult albino rats. Oral administration of 50 and 200 mg/kg b.w/animal/day of the extract for 1, 2, 4, and 12 weeks caused duration- and- dose-dependent changes in the body weights, organ weights and sperm characteristics. However, there was a gradual increase in the weights of the liver of all treated rats which was significant ($P < 0.05$) in those treated for 12 weeks. Sperm viability, motility and counts were significantly reduced ($P < 0.05$) in rats treated for 2 and 4 weeks. Only sperm viability was reduced in rats treated for 1 week. However, rats treated for 12 weeks had normal sperm motility, viability and counts. In addition, they had normal serum testosterone concentration and fertility. Fertility was zero in rats that were treated for 2 weeks. Visible lesions in the seminiferous tubular cytoarchitecture were observed in the histological sections of the testes from the treated rats prepared at the end of the study period. Normal sperm characteristics were however, restored in rats that were allowed 4 weeks recovery period. The results therefore suggest that the extract could have reversible antifertility effects in male rats.

Keywords
Alstonia boonei, Antifertility, Antimalarial, Male Rats

INTRODUCTION

Alstonia boonei is a tropical plant reputed in traditional medicine to have antimalarial, antipyretic, analgesic and anti-inflammatory properties (Ojewole, 1984; Olajide *et al*, 2000). Many antimalarial and antibiotic agents have been reported to have antifertility actions. For instance, the antisteroidogenic and antifertility actions of quinine, tylosine and chloroquine have been well documented (Sairam, 1978; Meisel *et al*, 1993; Adeeko and Dada, 1998).

The induction of reversible male infertility in experimental animals and humans resulting from treatment with medicinal plants and their products has also drawn the attention of researchers. The antisteroidogenic and antifertility activities of extracts from *Carica papaya, Quassia amara* and *Azadirachta indica* all of which have documented antimalarial properties have also been reported (Lohiya *et al*, 1994; Raji and Bolarinwa, 1997; Raji *et al,* 2003). With the increased efforts in the development of more potent antimalarial agents as a result of the challenge posed by the resistant strains of the malarial parasite, the evaluation of these antimalarial agents for possible antifertility actions becomes worthwhile. This is in view of the fact that both malaria and infertility are worldwide phenomena and the need to avoid the risk of infertility resulting from malarial chemotherapy. In the absence of information on the reproductive toxicity of *Alstonia boonei*, a potent antimalarial plant, the present investigations were therefore undertaken to determine the male reproductive effects of the methanolic extract of *Alstonia boonei* stem bark in rats.

MATERIALS AND METHOD

Animal Model: Wistar strain albino rats (150-240g) obtained from the Central Animal House, College of Medicine, University of Ibadan, were used for the study. The rats were housed in wire mesh cages under standard conditions (temperature 25-29 ^{o}C, 12h light and 12h darkness cycles). Animals were fed with pelleted standard rat diet (Ladokun feeds ltd, Nigeria)

and water *ad libitum*. Generally, the study was conducted in accordance with the recommendations from the declaration of Helsinki on guiding principles in care and use of animals.

Plant material: The stem bark of *Alstonia boonei* was collected from the campus of the University of Ibadan and identified at the Forestry Research Institute of Nigeria. The shade-dried stem bark was reduced to a powdery form by grinding and the powdered sample was exhaustively extracted with methanol. The solvent was then distilled off, to give the solid extract, which was stored at 4°C. The solid extract was then dissolved in normal saline and used for this study.

Experimental design: One hundred and twenty (120) rats were divided into 20 groups of six animals each. Groups 1 – 4 were normal control rats, received normal saline alone as vehicle for the extract. Groups 5 – 8 and 9 – 12 were treated with 50mg/kg and 200mg/kg body weight of *Alstonia boonei* extracts respectively for 1,2,4 and 12 weeks. Groups 13 – 16 and 17 – 20 represent the recovery groups (i.e. rats that were treated with 50mg/kg and 200mg/kg body weight of the extracts for 1, 2, 4 and 12 weeks respectively and which were allowed to recover for the same periods of time). Vehicle and extract administration were done orally.

Body and organ weights: Initial and final body weights of the animals were recorded. At the end of the one, two, four or twelve weeks, animals were sacrificed under ether anaesthesia 24h after the last dosing of the respective treatment duration. The testes, hearts, liver and kidneys were removed and weighed.

Sperm motility, viability, counts and morphology: The caudal epididymis was dissected out; an incision (about 1 mm) was made in the caudal epididymis. Sperm fluid was then squeezed onto the microscope slide. Epididymal sperm motility was assessed by calculating motile spermatozoa per unit area and was expressed as percent motility. Epididymal sperm counts were made using the hemocytometer 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 2 and 12 weeks were introduced to parous females in the ratio 1:2 for a period of seven days and the number of litters resulting from the cohabitation was recorded.

Serum biochemistry
Blood was obtained by cardiac puncture from the rats in each study group after anaesthesize them with ether. Each blood sample was spun at 2500 rpm for 10 minutes in a desktop centrifuge at 10-25 °C. Serum samples were assayed for testosterone using the enzyme linked immunoassay (EIA) technique.

Histological Processing: This was done as essentially as described by Akpantah *et al* (2003). 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 °C. Serial sections were cut using rotary microtome at 5 microns. Slides were prepared from these tissues. The slides were dewaxed 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 *t*-test and ANOVA where necessary. $P < 0.05$ was accepted as significant.

RESULTS

Body weight changes

The extract did not have any significant effect on the body weights of rats treated for 1 week and 2 weeks while the body weights decreased significantly (P<0.05) in those treated for 4 and 12 weeks. (Fig. 1). However, normal body and organ weights were restored in rats that were allowed a recovery period of 12 weeks.

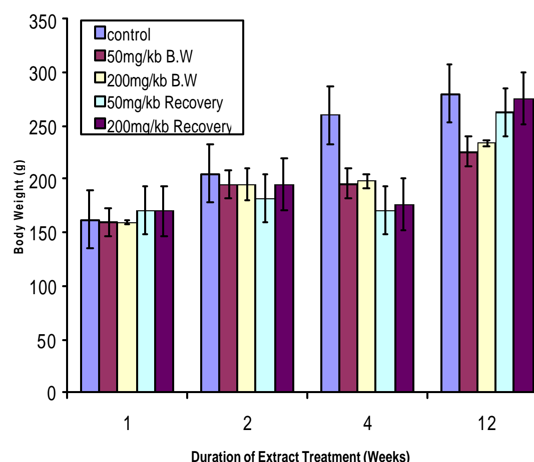


Fig. 1
Body weight changes in control rats and rats treated with Methanol extracts of *Alstonia boonei* and their recovery groups.

Table 1:
Effects of *Alstonia boonei* extracts on sperm characteristics in ratstreated for 1 week and their recovery group.

Groups	Motility (%)	Viability (%)	Sperm Counts (million /ml.)
Control	75.00 ± 20.03	85.00 ± 6.50	85.00 ± 25.00
50mg/kg	55.00 ± 15.04	50.00 ± 0.00*	80.00 ± 20.06
200mg/kg	70.00 ± 10.03	78.00 ± 7.52	53.00 ± 3.01
50mg/kg (Rec.)	55.00 ± 5.01	65.00 ± 5.01*	60.00 ± 5.52
200mg/kg (Rec.)	55.00 ± 5.01	65.10 ± 5.01*	51.00 ± 1.00

- *P<0.05*

Table 2:
Effects of *Alstonia boonei* extracts on sperm characteristics in rats treated for 2 weeks and their recovery groups.

Groups	Motility (%)	Viability (%)	Sperm Counts (million/ml).
Control	88.00 ± 7.52	90.00 ± 5.01	75.00 ± 6.02
50mg/kg	40.00 ± 10.03*	55.00 ± 5.01	58.00 ± 2.51*
200mg/kg	2.50 ± 0.00**	2.50 ± 0.00**	51.00 ± 1.00*
50mg/kg (Rec.)	5.00 ± 1.00**	5.00 ± 1.00**	20.00 ± 3.94*
200mg/kg (Rec.)	35.00 ± 5.00*	54.00 ± 7.47*	53.00 ± 2.69*

**P<0.05; **P<0.001.*

Table 3:
Effects if *Alstonia boonei* extracts on sperm characteristics in rats treated for 4 weeks and their recovery groups.

Groups	Motility (%)	Viability (%)	Sperm Counts (million/ml)
Control	60.00±2.89	68.00±2.51	57.00±5.01
50mg/kg	14.00±2.68**	46.00±3.99*	54.00±1.29
200mg/kg	28.00±7.99*	54.00±5.09*	54.00±1.22
50mg/kg(Rec.)	53.00±3.23	64.00±2.40	89.00±5.16
200mg/kg (Rec.)	50.00±10.47	48.00±5.82*	75.00±13.94

**P<0.05. **P<0.001.*

Table 4:
Effects of *Alstonia boonei* extracts on sperm characteristics in rats treated for 12 weeks and their recovery groups.

Groups	Motility (%)	Viability (%)	Sperm Counts (million/ml).
Control	92.00±1.22	93.00±1.29	95.00±7.47
50mg/kg	67.00±18.58	68.00±19.24	94.00±11.39
200mg/kg	71.00±12.67	72.00±12.08	87.20±8.84
50mg/kg	89.50±1.20	90.00±2.50	88.00±3.50
200mg/kg (Rec.)	90.20±2.50	92.00±3.20	90.50±2.20

Organ weight changes
Except in rats treated for 12 weeks, there was no significant change in the weights of testes, liver and hearts of the treated rats. Normal organ weights also appear to be restored in rats treated with 50mg/kg but not in those treated with 200mg/kg in the other treatment duration (data not shown).

Sperm motility
The sperm motility was significantly reduced (P<0.001) in rats treated for 2 and 4 weeks whereas there was no significant change in the motility of those treated for 1 week and 12 weeks. The decrease in motility was dose-dependent in the rats that were treated for 2 weeks where 40% and 2.5% were recorded for rats treated with 50mg/kg and 200mg/kg respectively (Tables 1 – 4).

Sperm viability
The sperm viability reduced significantly (P<0.05) in rats that were treated with 50mg/kg body weight but there was no change in those treated with 200mg/kg for 1 week. A significant reduction (P<0.001) was also observed in the viability of sperm in the rats treated for 2 weeks and also those that were treated for 4 weeks (P<0.05). The changes in the sperm viability were both duration – and dose-dependent. Sperm viability was significantly higher (P<0.001) in 50 mg/ kg rats than in those treated with 200mg/kg of the extracts for 2 weeks. There was, however, no significant difference in sperm viability of rats treated with each of the doses of the extract for 4 and 12 weeks (Tables 1 – 4).

Sperm Counts

There was no significant difference in the sperm counts of the rats that were treated for 1,4 and 12 weeks when compared with their control counterparts. However, a significant reduction (P<0.05) was observed in the sperm counts of rats treated for 2 weeks. Moreover, no significant difference was observed in the sperm counts of rats that were treated with each of the doses of the extract for 1,4 and 12 weeks. However, in rats treated for 2 weeks, the sperm counts were significantly higher (P<0.05) in those treated with 50mg/kg than those treated with 200mg/kg. (Tables 1 – 4).

Morphology.

The most common abnormality of the epididymal sperm was "curved mid piece", which accounted for 28.5%, 19.2%, 27% and 20% of the total abnormality in the rats treated for 1, 2, 4 and 12 weeks respectively. The abnormalities observed were mainly secondary and tertiary. However, primary abnormalities characterized by "small and pyriform heads" were observed only in rats that were treated for 2 weeks.

Serum Testosterone Level

There was no significant difference in the serum levels of testosterone in rats treated with 50mg/kg and 200mg/kg of the extract for 12 weeks when compared with their control counterparts. The serum testosterone concentration was 1.80 ± 0.20 ng/ml in the control rats while it was 1.0 ± 0.80 and 1.30 ± 0.50 ng/ml respectively in those treated with 50 mg/kg and 200mg/kg of the extract (Fig. 2)

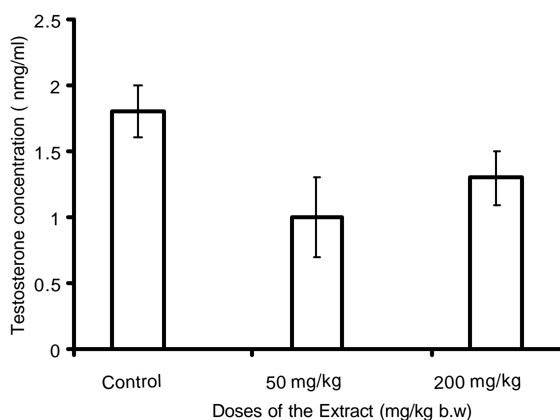


Fig. 2
Effects of Alstonia boonei extract on serum testosterone level in rats

Histology

There were visible lesions in the testes of *Alstonia boonei* treated rats when compared with their control counterparts. These were characterized by moderate-to-mild degeneration of the seminiferous tubular epithelium, vascular congestion, interstitial oedema and degeneration of the interstitial cells of Leydig. These lesions were, however, severe in the rats that were treated for 2 weeks. Moreover, there was no significant change in the histological features of the testes in rats that were treated for 12 weeks (Plates 1 – 4).

Fertility

The fertility of rats that were treated for 12 weeks with both 50mg/kg and 200mg/kg body weight of the extract was not affected. The rats impregnated all the female rats cohabited with them within a period of seven days and the number of litters resulting from the cohabitation was not significantly different from that of their control counterparts. The value of 4.50 ± 0.50 was recorded for the control rats while 4.00 ± 1.00 and 3.50 ± 0.50 were recorded for those treated with 50mg/kg and 200mg/kg body weight of the extract respectively. However, fertility was zero in rats that were treated for 2 weeks.

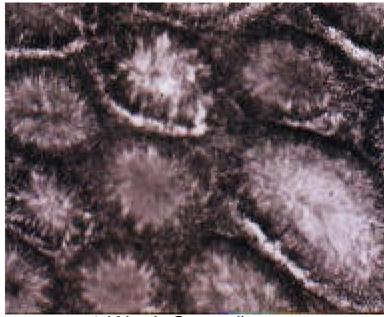
Recovery

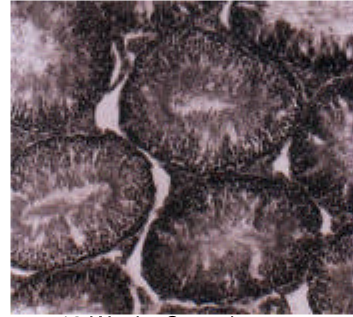
In the recovery groups, the sperm motility remained normal in rats that were treated for 1 and 12 weeks, as it was unchanged in rats that were not allowed to recover. The reduction in sperm motility in the rats treated for 4 weeks returned to normal in their recovery groups while the reduction in sperm motility in rats treated for 2 weeks failed to return to normal in their recovery groups. The sperm viability also remained normal in rats that were treated for 12 weeks while it was still significantly reduced in rats treated for 1 week (P<0.05) and 2 weeks (P<0.001). The sperm viability, however, returned to normal in rats that were treated with 50mg/kg of the extract for 4 weeks and which were allowed to recover for the same period of time but it was not restored in those treated with 200mg/kg.

The sperm counts also remained normal in the recovery groups of 1 and 12 weeks while it was still significantly reduced (P<0.05) in the recovery groups of 2 weeks. It was however, significantly higher (P<0.05) in the recovery group of 4 weeks. There was also an apparent restoration of normal histological features in the testes of the recovery groups except in the recovery groups of 2 weeks in which severe lesions still persisted (Tables 1 – 4).

DISCUSSION.

The results from these studies suggest that *Alstonia boonei* could cause reproductive impairment in male rats. The decrease in the body weights of rats treated for 4 and 12 weeks could be due to the activities of echitamine and echitamidine, potent constituents of *Alstonia boonei*, which have earlier been reported to have diuretic and hypotensive properties (Kucera *et al*, 1972; Maurice, 1993).

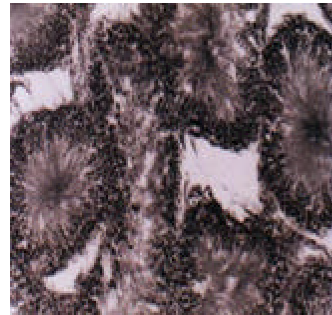
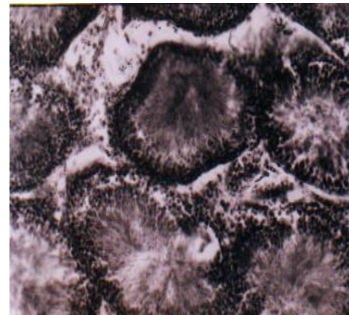




1 Week Control)   2 Weeks Control   12 Weeks Control

Plate 1.

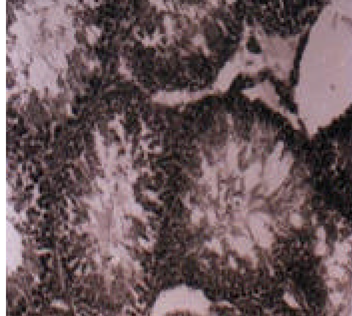
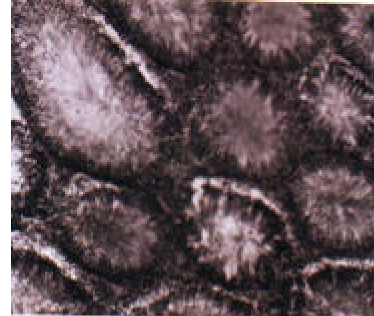
Transverse sections through the Testes of normal control rats for 1, 2, 4 and 12 weeks *Alstonia boonei* treated rats. The seminiferous tubules (single arrow) and Leydig (double arrows) remained intact (Mag. X 160).

1 Week 50 mg/kg extract treated   2 Weeks 50 mg/kg extract treated   12 Weeks 50 mg/kg extract treated

Plate 2:

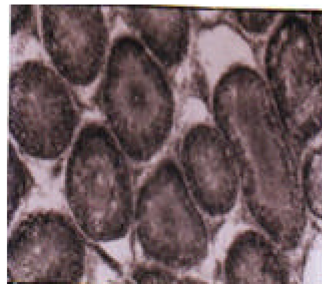
Sections through the testes of rats treated with 50mg/kg body weight of the extract for 1, 2, 4 and 12 weeks respectively. There were moderate to mild vascular congestions and seminiferous tubular degeneration. (X 160)

1 Week 200 mg/kg extract treated   2 Weeks 200 mg/kg extract treated   12 Weeks 200 mg/kg extract treated

Plate 3:

Sections through the testes of rats treated with 200mg/kg body weight of the extract for 1, 2, 4 and 12 weeks respectively. Mild vascular congestions and degeneration of the seminiferous tubular epithelium were visibly prominent in L, M, N (X 160).



1 Week (Recovery)   2 Weeks (Recovery)   12 Weeks (Recovery

Plates 4:

Transverse sections through the testes of the recovery groups of rats treated with 200 mg/kg body weight of the extract for 1, 2, 4 and 12 weeks respectively. The seminiferous tubular epithelium had been regenerated. Vascular congestion, interstitial oedema and degeneration of the Leydig cells persisted in plate 17 (X 160).

The diuretic activities of these constituents could have resulted in loss of water and electrolytes and consequently, loss in body weights. The significant decrease in the testicular weights of rats treated with 50mg/kg of the extract for 12 weeks could not be readily accounted for as there was no significant decline in the serum levels of testosterone and sperm characteristics in the rats when compared with their control counterparts. Testosterone is required for the growth, development and maintenance of male reproductive organs (Mooradian *et al*, 1987).

The significant decrease in the sperm motility of rats that were treated for 2 and 4 weeks suggest that the extract was able to permeate the blood-testis barrier. The decrease in sperm motility caused by chemical agents had earlier been reported to be due to their ability to permeate the blood-testis barrier (Baldessarini, 1980) and thus, creating a different microenvironment in the inner part of the wall of the seminiferous tubules from that in its outer part (Bloom and Fawcett, 1975).

Although, the activity of lactate dehydrogenase, the enzyme that catalyzes the reversible reaction between lactate and pyruvate was not evaluated in this study, the activi ty of the enzyme has been reported to be related to sperm counts and motility (Gerez de Burgos *et al*, 1979) as a result of its involvement in energy-supplying metabolic processes. It is however tempting to suggest that the activity of the enzyme, a protein, could also be affected by the active constituents in the extract the same way the Hb concentration was affected. This is reasonable in view of the fact that the Hb also has a protein component and in either case, the result is the alteration in the production of pyruvate and energy, which could alter sperm activity and survival.

It is noteworthy that there was no significant decline in serum levels of testosterone in 12 weeks *Alstonia boonei*-treated rats. The significant difference in the sperm motility, viability and counts of these rats provides evidence for the significant alterations in the circulating androgen levels. Testosterone is required for the growth and development of male reproductive organs (Mooradian *et al*, 1987) and in association with follicle stimulating hormone, acts on the seminiferous tubules to initiate and maintain spermatogenesis (Christensen, 1975). These studies indicate that *Alstonia boonei* is probably capable of reducing serum levels of testosterone and further suggest that it could have deleterious effects on testicular androgen secretory function. The degeneration of Leydig cells in rats treated for 2 and 4 weeks, suggests that the extract could have a direct effect on the Leydig cells steradogenesis in-vivo. In the recovery groups, however, only rats that were allowed to recover for 4 weeks recovered fully while those on 2 weeks recovery did not. The full restoration of sperm performance in these rats is an indication that the rats would require at least 4 weeks before the effects of the extract could be fully removed from them.

Although, there have been no reports on the metabolism of *Alstonia boonei*, the restoration of sperm performance in the rats that were allowed to recover for 4 weeks appears to suggest that the active constituents might have been metabolized and excreted after 4 weeks. This coupled with the diuretic effects of echitamine and echitamidine, potent constituents of *Alstonia boonei*, which could probably increase the clearance rate of the extract coul d have been responsible for the inefficacy observed in rats treated for 12 weeks which had all variables similar to their control counterparts. It may also be due to the development of resistance induced by long-term treatment.

The normal fertility in rats treated for 12 weeks could be attributed to normal sperm characteristics in the rats while the zero fertility recorded in those treated for 2 weeks could also be due to the significant reduction in sperm characteristics especially the sperm motility. Reduction in fertility and sterile mating recorded after the treatment of male rats with *Carica papaya* seeds and chloroquine has been reported to be due to the reduction and impairment in sperm motility (Lohiya *et al*, 1994; Adeeko and Dada, 1998). Immotile or sluggishly motile spermatozoa would not penetrate the cervical mucus and thus could fail to fertilize the ova. In addition, the libido of the rats treated for 2 weeks could have been affected, as they were less aggressive for untreated female rats when compared with those treated for 12 weeks. The fertility would also be reduced in rats treated for 4 weeks in view of their significant reduction in motility while normal fertility is expected in those treated for 1 week and those allowed to recover for 4 weeks.

The results suggest that the extract could have deleterious effects on sperm performance and fertility in rats that were treated for 2 and 4 weeks. The adverse effects were probably mediated via reductions in sperm metabolism, serum testosterone concentration and probably direct spermatoxic effects. These effects appear to be reversible four weeks after the withdrawal of the extract treatment.

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