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Quinolizidine alkaloids: the bioactive principles in *Cnestis ferruginea* (de Candolle) with male antifertility activities

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Summary

Objectives: The reproductive activities of purified fractions from the root extract of *Cnestis ferruginea* was evaluated in rats. Phytochemical screening of the extract revealed the presence of alkaloids, tannins and anthraquinones.

Materials and Methods: Column chromatography produced 20 fractions which were reduced to 6 by thin layer chromatography. Nuclear magnetic resonance spectroscopy revealed quinolizidine alkaloids (fractions 3 and 4) as the active principles in the extract of *Cnestis ferruginea*. Each fraction of *Cnestis ferruginea* (0.1, 1 and 2 $mgkg^{-1}bw$) was administered to rats by gavages for 60 days.

Results: All fractions caused significant reduction ($p<0.05$) in sperm counts, motility, viability, morphology and plasma levels of testosterone, luteinizing hormone and follicle stimulating hormone. Fractions 3 and 4 caused the highest reduction ($p<0.001$) in fertility, FSH and LH levels comparable to those of quinine sulphate. There was recovery after 60 days of withdrawal from the extracts.

Conclusion: The results suggest that *Cnestis ferruginea* possesses reversible male antifertility effects. The active principles with these activities appear to be quinolizidine alkaloids.

Keywords: *Antifertility, Cnestis ferruginea, male, quinolizidine, reproduction*

Résumé

Les activités reproductive des fractions d'extraits purifies de *Cnestis ferruginea* était évalué sur les souris blanc. Un dépistage phytochimiques des extraits révélaient la présence des alcaloïdes, tannins et anthraquinones. La chromatographie de colonne produisait 20 fractions qui étaient réduit à 6 par la chromatographie sur papier léger. La spectroscopie à résonance nucléaire et magnétique révélait des alcaloïdes quinolizidine (fractions 3 et 4) comme principe actif dans l'extrait de *Cnestis ferruginea*. Chaque fraction de *Cnestis ferruginea* (0.1, 1 et 2 $mgkg^{-1}bw$) était administrée aux souris par gavages pour 60 jours. Toutes les fractions causaient une réduction significative ($p<0.05$) des quantités de spermes, motilité, viabilité, morphologie et les taux de plasma du testostérone, hormone lutéinique et follicule stimulating hormone. Les fractions 3 et 4 causaient la plus grande réduction ($p<0.001$) en fertilité, en taux de FSH et LH comparable à celles qui recevaient de la quinine de sulfate. Il y avait une convalescence après 60 jours de retrait des extraits. Les résultats suggèrent que le *Cnestis ferruginea* possède des effets d'infertilité réversible chez l'homme. Les principes actifs avec leurs activités paraient être des alcaloïdes de quinolizidine.

Introduction

Cnestis ferruginea belongs to the family of *Connaraceae* plants and is highly ubiquitous in the southern part of Nigeria. It is known as *omu aja or gboyin gboyin* in Yoruba, *amunketa* in Igbo, *Utina bua* in Efik and *Ukpe-ibieka* in Bini tribes of Nigeria. *Cnestis ferruginea* is known to possess powerful antibiotic activities; extract of whole plant of *Cnestis ferruginea* has been used to treat conjunctivitis, syphilis, gum pain, wounds, dysentery and gonorrhea. The traditional medicine practitioners in Nigeria use the root decoction of the plant to stabilize pregnancy and to treat ovarian disorder [1].

It is important to note that many antimicrobial agents possess male anti-fertility activity in varying degrees. For instance chloroquine, quinine, quinacrine, nitrofurans, erythromycin, tylosin and tetracycline have been reported to cause varying degrees of steroidogenic, spermatogenic and fertility inhibition in man and animal models [2, 3, 4, 5]

Interestingly many antimalarial and antibacterial medicinal plant extracts have also been reported in experimental male infertility. For example, *Quassia amara* which was reported to be highly potent against chloroquine resistant *Plasmodium falciparum* [6] produced significant reduction in epididymal sperm counts, serum levels of testosterone, luteinizing hormone (LH) and follicle stimulating

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hormone (FSH) in male rats [7,8,9,10]. *Alstonia boonei,* a tropical plant, reputed in traditional medicine to have antimalarial, antipyretic, analgesic and anti-inflammatory properties, was reported to cause duration- and- dose-dependent changes in the body weights, reproductive organ weights and sperm viability, motility and counts [11]. *Azadirachta indica,* a medicinal plant with potent antiplasmodium activities in mice was reported to cause mass atrophy of the spermatogenic elements and Leydig cells when administered to male rats [12,13, 14,15]. Similarly, antimicrobial medicinal extracts from *Morinda lucida, Sphenocentrum jollyanum, Carica papaya* and *Ricinus communis* [16,17,18,19] have been reported to cause antifertility activities in rats. The major bioactive component of the aforementioned medicinal plants including *Cnestis ferruginea* is alkaloid.

There is however no information in the literature on the reproductive activity of *Cnestis ferruginea.* In our preliminary study daily oral administration of methanol extract of *Cnestis ferruginea* caused a reversible reduction in sperm count, viability, motility, and morphology, weight of the testis and plasma testosterone [38]. These findings prompted us to investigate in more detail the reproductive activities and the possible mechanism of action of the purified fractions of *Cnestis ferruginea* in male albino rats.

Materials and methods

Plant material and methanol extraction

Root of *Cnestis ferruginea* was collected at the botanical garden, University of Ibadan, Ibadan Nigeria, where it was authenticated. A voucher specimen (UIH 22272) was deposited at the herbarium of the Department of Botany and Microbiology, University of Ibadan. The root of *Cnestis ferruginea* was air dried and pulverized before the commencement of the methanol extraction. The extraction was carried out as earlier described [7]. The pulverized root (about 2,750g) was exhaustively extracted with methanol by means of Soxhlet apparatus and the extract evaporated *in vacuo.* The root extract of *Cnestis ferruginea* was concentrated *in vacuo* using a rotary evaporator. The solvent (methanol) remaining in the extract was removed by placing the extract in porcelain dishes in temperature-controlled oven to give a residue weighing 25g (0.91% yield).

Purification of the crude extract by chromatography

A modified form of classical glass column chromatography (flash chromatography) was used for the purification of the root extract of *Cnestis ferruginea* in this study. The mobile phase consisted of 3 solvents; hexane (non-polar), ethylacetate (mid-polar) and methanol (polar). The various proportions of solvents were passed through the bed by the application of positive pressure using the vacuum pump. Twenty fractions were obtained. Separation of column fractions was then done by thin layer chromatography (TLC). The TLC plate was examined under (354nm) ultraviolet light and any spots visualized with this procedure in order to locate the compounds in the sample. With this procedure 6 fractions were finally obtained.

Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectroscopy was carried out using the Varian-Mercury NMR Spectrophotometer (Varian Associates, UK) operating at 200MHz for proton (50MHz for carbon nuclei and 50 MHz for ^{13}C). The positions of proton and carbon resonance in the NMR spectrum were measured relative to the resonance position of tetramethylsilane (TSM) as internal standard. The NMR spectrum of the pure compound was carried out using a 200MHz machine for 10% (w/v) solution in deuteriomethanol. The pulse irradiation technique employed was FT NMR at ambient temperature.

Animals

Adult male Wistar albino rats obtained from the Central Animal House, College of Medicine, University of Ibadan, were used for the experiments. They were certified fertile by isolated mating technique. They were then acclimated to laboratory conditions (12 hours dark-light period), housed five per cage and fed with rat cubes (Ladokun feeds limited, Ibadan, Nigeria) and water *ad libitum.* The weight range of the rats was 160-220grams before the commencement of the study. The study was conducted in accordance with the recommendations from the declaration of Helsinki (as revised in Tokyo 2004) on guiding principles in care and use of animals.

Experimental design

Administration of pure fractions of Cnestis ferruginea

The six purified fractions of *Cnestis ferruginea* were used in this study. Fifteen male rats divided into three equal groups were administered orally

0.1, 1 and 2 mgkg^{-1}bw of fraction1 of Cnestis ferruginea for 60 days. The same protocol was applied to fractions 2, 3, 4, 5, and 6 of Cnestis ferruginea with 15 rats in each fraction. The control group received 0.5ml of distilled water (vehicle for the Cnestis ferruginea extract). At the end of the treatment period each rat was bled through the orbital sinus. The blood sample collected was centrifuged at 3,000 r.p.m. for 10 mins and plasma was obtained for measurement of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone levels.

Hormone assay

This was carried out as earlier described [18]. An enzyme-based immunoassay (EIA) system was used to measure LH, FSH and testosterone levels in plasma samples collected. The EIA kits were obtained from Immunometrics, London, UK. LH EIA kit ((Product Code IM101), FSH kit (Product Code IM102) and testosterone kit (Dialab Catalog No K00234) and contained specific hormone EIA enzyme label, EIA substrate reagent and EIA quality control sample. A quality control was carried out at the beginning and the end of the assay to ascertain the acceptability with respect to bias and within batch variation.

Sperm collection and analysis

Sperm motility, viability, morphological characteristics and epididymal sperm count were carried out [18]. Briefly, the caudal epididymis was removed and quickly transferred to a pre-warmed slide (27 °C) and lacerated with a razor. Sperm was squeezed onto the microscope slide and two drops of warm 2.9 % sodium citrate was added. This was then covered with a cover slip, examined and scored for sperm motility under the microscope using the ×40 objective of the microscope. A viability study (percentage of live spermatozoa) was done using the eosin/nigrosin stain. Sperm was squeezed onto a microscope slide and two drops of the stain was added. The motile (live) sperm cells were stained. The stained and the unstained sperm cells were counted using ×40 objectives of the microscope and an average for each was taken from which percentage viability was calculated. Sperm morphology was done by staining the sperm smears on microscope slides with two drops of Wells and Awa stain and air-dried [37]. The slides were examined under the microscope using ×100 objectives under oil immersion. The abnormal sperm cells were counted and the percentage calculated. Sperm count was done under a microscope with the aid of the improved Neubauer hemocytometer.

Mating experiment

Isolated mating technique was adopted in this study for only the group that received 1 mgkg^{-1}bw. Immediately after the last dosing (1 mgkg^{-1}bw) of each fraction, a male rat from each of the 7 groups (the 6 fractions and the control groups) was cohabited with 3 untreated female rats (each weighing about 200g) of proven fertility for one week. Vaginal smear was carried out daily and successful insemination was confirmed by the presence of spermatozoa in the smear. The day of detection of spermatozoa and vaginal plug was taken as day one of pregnancy.

Recovery experiment

A group of 5 rats were treated with 1mgkg^{-1}bw of *Cnestis ferruginea* for 60 days and allowed a recovery period of 60 days. Each rat was then killed by cervical dislocation. Sperm analysis was carried out. The seminal vesicles, epididymides, testes, liver and heart were removed and weighed.

Studies on the bioactive fractions of Cnestis ferruginea and quinine sulphate

The standard alkaloid used for this study was quinine sulphate ($C_{20}H_{24}N_2O_2)2.H_2SO_4.2H_2O$) (BDH Chemical Ltd Poole, England). Fifteen rats were divided into 3 equal groups with group 1, receiving 20mgkg^{-1}bw; group 2, 40mgkg^{-1}bw intramuscularly for 7 days while group 3 (control) received distilled water based on a previous study [5]. Sperm was collected from the caudal epididymis after sacrificing the rats for the analysis of sperm functions. The activity of quinine was compared with the fractions (3 and 4) of *Cnestis ferruginea.*

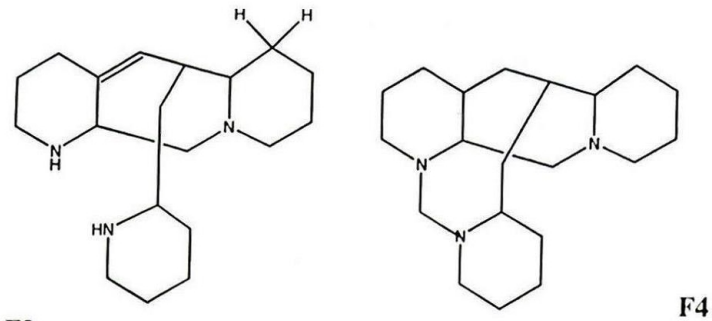
Statistical analysis

Mean values and the standard deviation (Mean ± SD) were calculated. The test of significance between two groups was determined by Student's *t* test [20] and for more than two groups by the analysis of variance (ANOVA) with Duncan's multiple range tests [21].

Results

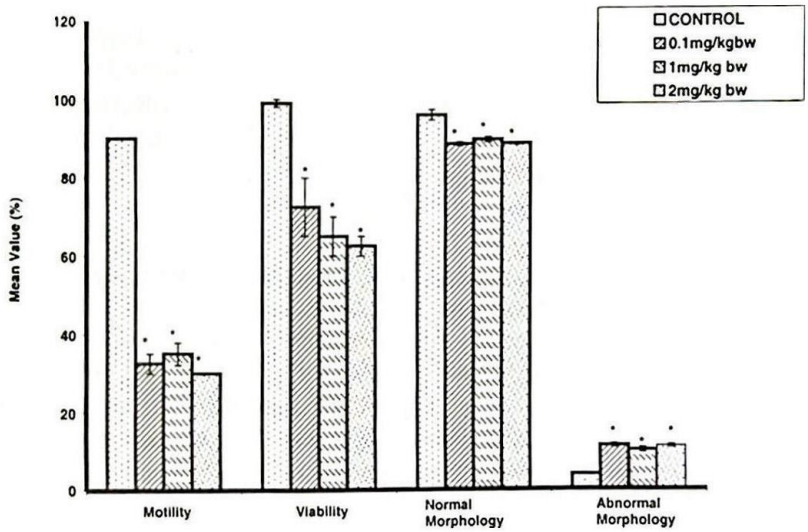
Proposed structures of quinolizidine alkaloids in Cnestis ferruginea

The ^{1}H spectrum revealed the presence of aliphatic protons and a few deshielded protons. No aromatic protons were detected, except for some highly deshielded broad band protons at around 7.00ppm. The ^{13}C decoupled spectra showed the presence of 52 carbon atoms including a carboxylic carbon at 163.269ppm. Some of carbon signals are embedded in the signals due to deuterated methanol. The ^{1}H and



F3 F4

Fig. 1: Proposed structures of quinolizidine alkaloids in *Cnestis ferruginea* from NMR spectroscopy analysis



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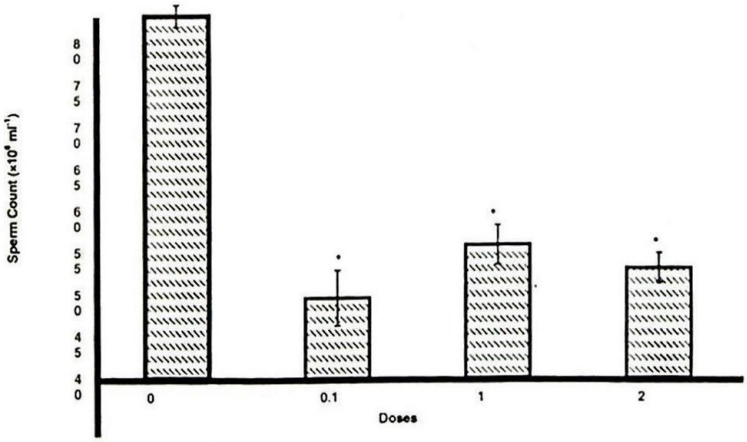

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Fig. 2: Effect of Fraction 3 of *Cnestis ferruginea* on sperm functions of male rats. Values are expressed as Mean±SEM. Values significantly different from control (*$p<0.01$)

^{13}C spectra relate closely with the description earlier reported [22] to describe a series of quinolizidine alkaloids found in *Connarus paniculatus var paniculatus* Roxb, another member of *Connaraceae.* Thus, the spectra obtained appear like a mixture of two of these alkaloids. The proposed structures of the quinolizidine alkaloids in F3 and F4 are as shown in Fig. 1.

Individual effect of the six pure fractions of Cnestis ferruginea on sperm functions

As shown in Figs. 2 and 3, fractions 3 and 4 of the pure extract of *Cnestis ferruginea* (F3, F4) caused significant decrease ($p<0.01$) in sperm counts, motility and viability when compared with the control. The number of abnormal sperm were significantly higher ($p<0.01$) in all the extract treated rats than in the control group. These results were similar but with mild effects in fractions 1, 2, 5 and 6.

group was not significantly different ($p>0.05$) from the litter sizes of females mated with F1, 2, 5 and 6 male rats. The litter size of the control group was however significantly higher ($p<0.001$) than the litter sizes of female rats mated with male rats that were treated with F3 and F4.

Table 1: Effect of Fractions 3 and 4 of *Cnestis ferruginea* on testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels in male rats. Values are expressed as Mean±SEM

Hormone	Doses of F3 and F4 (mg/kg bw)	0	0.1	1	2
Testosterone	F3	10.00±0.41	0.70±0.05*	0.5±0.02*	0.40±0.02*
(ng/ml)	F4	10.00±0.39	0.40±0.02*	0.4±0.03*	0.20±0.01*
LH (IU/L)	F3	0.22±0.01	0.14±0.01*	-	-
	F4	0.25±0.01	0.07±0.01*	-	-
FSH (IU/L)	F3	0.25±0.01	0.05±0.01*	-	-
	F4	0.10±0.01	0.03±0.01*	-	-

*Values significantly different from control (*p<0.001*

Effect of the six pure fractions of Cnestis ferruginea on reproductive hormones

As shown in Table 1 the three doses (0.1, 1 and 2 $mgkg^{-1}bw$) of F3 and F4 significantly ($p< 0.001$) reduced plasma testosterone levels in rats. Both F3 and F4 of *Cnestis ferruginea* caused the highest significant reduction ($p<0.01$) in the levels of testosterone, LH and FSH. LH and FSH were undetectable at the doses of 1 and 2 mg/kg of F3 and F4. Similar but less significant results were obtained in F1, 2, 5 and 6 (data not shown).

Comparison of the effects of quinine and Fractions 3 and 4 of Cnestis ferruginea on sperm functions

As shown in Fig. 4, the effects of 20 $mgkg^{-1}bw$ quinine and 1 $mgkg^{-1}bw$ of F3 of the pure extract of *Cnestis ferruginea* on sperm functions were similar. Both quinine and *Cnestis ferruginea* (F3) significantly reduced ($p<0.001$) sperm count, motility and viability. The percentage of abnormal sperm was increased significantly ($p<0.001$) in both quinine and *Cnestis ferruginea* (F3) treatment groups. The percentage reduction in the sperm count for F3 and quinine were

Table 2: Effect of the six fractions of *Cnestis ferruginea* on litter size of female rats which were cohabited with *Cnestis ferruginea*-treated male rats. Values are expressed as Mean±SEM

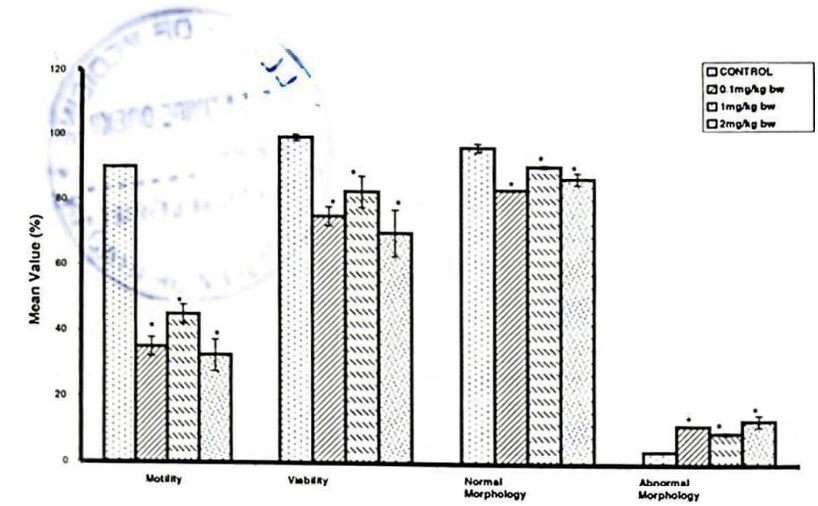
Fractions of *Cnestis ferruginea* (1mg/kg bw)	Control	F1	F2	F3	F4	F5	F6
Litter Size	7.33±0.50	7.00±0.00	6.67±0.58	4.33±0.58*	3.67±0.58*	7.00±1.00	6.67±0.58

Values significantly different from control (*p<0.001)

Individual effect of the six pure fractions of Cnestis ferruginea on fertility index

Table 2 shows the effect of the six fractions of *Cnestis ferruginea* on fertility of male rats in mating experiment. The litter size of female rats in the control

30% and 17%, respectively while the percentage reductions in sperm motility for F3 and quinine were 61% and 31%, respectively. The percentage reductions in sperm viability were 34% and 19% for F3 and quinine respectively. The percentage reduction



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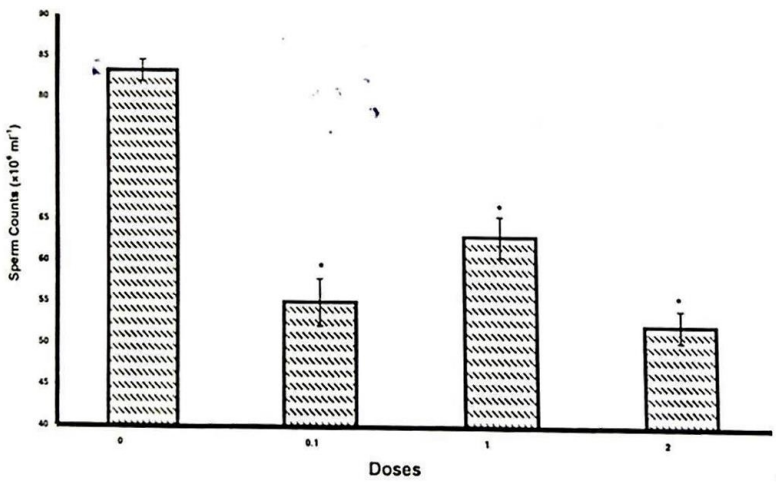

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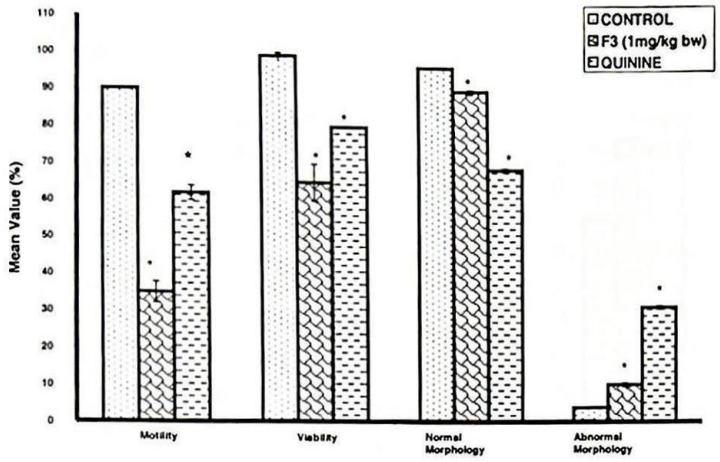
Fig. 3: Effect of Fraction 4 of *Cnestis ferruginea* on sperm functions of male rats. Values are expressed as Mean±SEM. Values significantly different from control (*p<0.01)

in sperm count for F4 and quinine were 18% and 17%, respectively. While the percentage reduction in sperm motility for F3 and quinine were 50% and 31%, respectively the percentage reductions in sperm viability were 11% and 19% for F4 and quinine respectively (Fig. 5). There was significant recovery in all the parameters measured.

Discussion

The extract of *Cnestis ferruginea* was purified using glass column and thin layer chromatography. The chemical constituents in the extract were characterized by NMR which identified quinolizidine alkaloids (fig.1) as the main compounds in the plant extract. The present study revealed that the quinolizidine alkaloids isolated from *Cnestis ferruginea* caused significant reduction in sperm functions. The negative impact of *Cnestis ferruginea* on sperm functions were however most noticeable in rats treated with fractions 3 and 4. This may be the reason why treatment with fractions 3 and 4 caused significant reduction in fertility of the male rats



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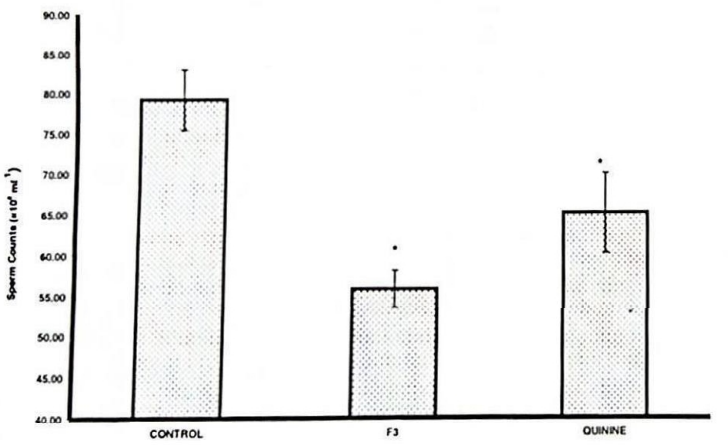

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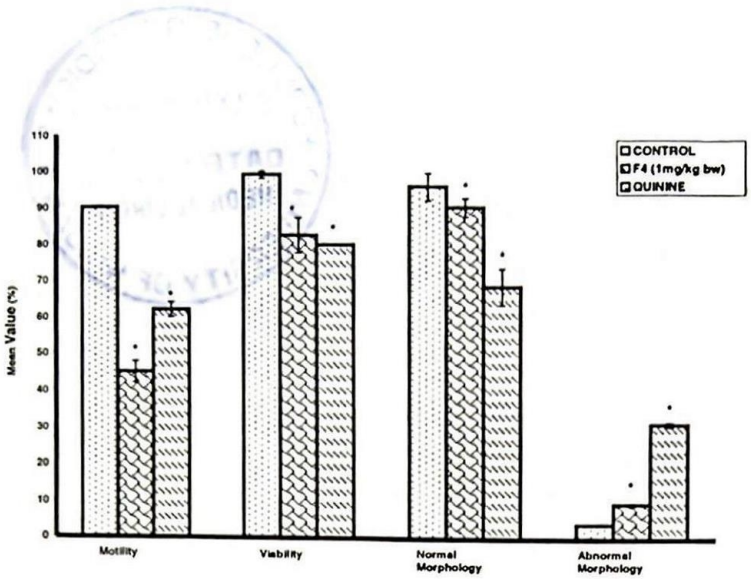
Fig. 4: Effects of quinine sulphate and F 3 of *Cnestis ferruginea* on sperm functions of male rats. Values significantly different from control (*p<0.001)

cohabited with female rats. Immotile or sluggishly motile spermatozoa would not penetrate the cervical mucus and thus could fail to fertilize the ova.

Cnestis ferruginea, whose main bioactive constituent is alkaloid has antiplasmodial activity against 3D7 strain of *Plasmodium falciparum* [23]. The plant was also reported to have anti-bacterial activities; the whole plant extract has been used to treat infections resulting in conjunctivitis, syphilis, gum pain, wounds and dysentery [1]. Many antimalarial and antibacterial agents have been reported to have antifertility actions. For instance, the antisteroidogenic and antifertility actions of quinine, tylosin and chloroquine have been well documented [2,3,4,5]. In the present study, there were also significant decreases in plasma testosterone levels of rats treated with purified extracts of *Cnestis ferruginea*. The actions of *Cnestis ferruginea* on male reproductive indices in this study are similar to those produced by *Carica papaya* seed extract [19], *Quassia amara* and quassin [8,9,10], *Azadirachta indica* [9,12,13,15] and *Alstonia boonei* [11]. It is therefore possible that the significant reduction in plasma testosterone levels of rats by the chromatographic fractions of *Cnestis ferruginea* in



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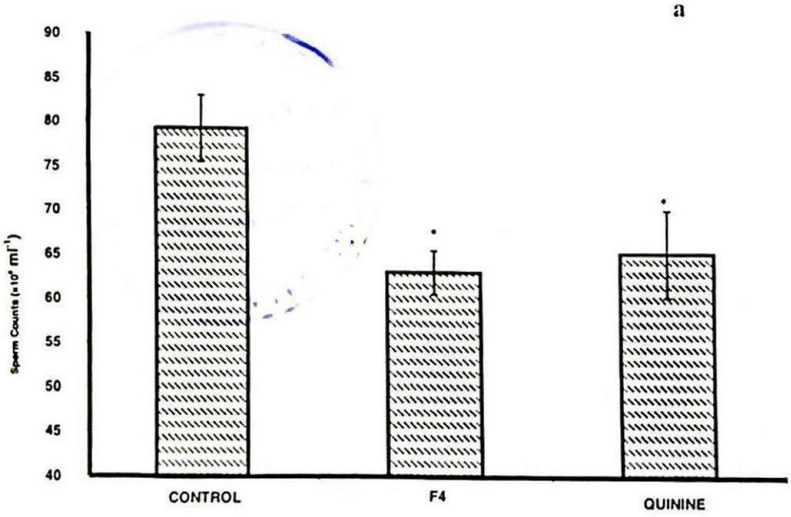

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Fig. 5: Effects of quinine sulphate and F 4 of *Cnestis ferruginea* on sperm functions of male rats. Values significantly different from control (*p<0.001)

the present study is due to the inhibition of testicular steroidogenesis by the combined action of the antimalarial and antibacterial properties of the plant. Several antimicrobial (antimalarial and antibiotic) agents including medicinal plants with antimicrobial activities have been reported to cause various degrees of antifertility effects in man and animal species [4, 16,17,18,19,24,25]. Furthermore, fractions 3 and 4 of *Cnestis ferruginea,* which seems to have the highest antifertility activity, also significantly reduced the plasma levels of LH and FSH in the treated rats. It is known that the building block of steroid hormone biosynthesis is cholesterol. The conversion of cholesterol to steroid hormones is stimulated by interstitial cell stimulating hormone [26], which leads to enzyme-directed conversion of cholesterol to steroid hormones. The lack of sufficient cholesterol or LH in the testis could impair these processes.

Spermatogenesis is influenced by the hypothalamic-adenohypophysial-Leydig cell system relating gonadotrophin releasing hormone (GnRH), LH and androgen. Therefore the decrease in sperm counts by the purified fractions of *Cnestis ferruginea* could be related to a decrease in plasma level of

testosterone and FSH because these hormones act synergistically in the initiation and maintenance of spermatogenesis [27]. FSH is known to act on the Sertoli cell in the seminiferous tubules and the importance of Sertoli cell in relation to spermatogenesis was due to the close intercellular association between them and spermatogonia. The decrease in sperm count and the high number of morphologically abnormal sperm caused by the fractions of *Cnestis ferruginea* indicate their interference with testicular spermatogenesis. It is also possible that the decline in sperm functions recorded in this study was a consequence of *Cnestis ferruginea* induced inhibition of testosterone secretion which is required for growth, development , maintenance and normal functioning of male reproductive organs [28].

Cnestis ferruginea might also act directly on the epididymis since various fractions of it caused significant reduction in sperm motility. After spermiogenesis, spermatozoa are moved through the *ductuli efferentis* to the caudal epididymis where they are stored prior to ejaculation. The wall of the epididymis contains smooth muscle and columnar epithelium which secretes the epididymal fluid that is essential for sperm survival, maturation, motility, capacitation and fertilizing ability [29]. The molecular basis for these processes is beginning to be unraveled through the discovery of some novel epididymis-specific genes and their functions [30,31,32,33].

Nuclear magnetic resonance (NMR) spectroscopy revealed that the active principles in F3 and F4 are quinolizidine alkaloids. Many medicinal plants contain alkaloids which have been reported to have antifertility effects. For instance, quinine, a natural white crystalline alkaloid with antipyretic, antimalarial and analgesic properties has been reported to have antispermatogenic activities [5]. In the present study it was observed that the antifertility activities of *Cnestis ferruginea* were similar to those of quinine. Solasodine, a steroidal alkaloid of *Solanum xanthocarpum,* was reported to induce antifertility in male rats [34]. Furthermore, vincristine, an indole alkaloid from *Vinca rosea,* was reported to cause male infertility [35]. Capine, which is an alkaloid from *Carica papaya,* caused severe degeneration of germinal epithelium and germ cells [19]. Embelin, an alkaloid isolate of *Embelia ribes* was observed to alter testicular histology in the male rats leading to the interference of spermatogenesis [36]. Therefore, the observation in the present study that an alkaloid is responsible for the antifertility activities of *Cnestis ferruginea* is consistent with previous findings on the toxicity of alkaloids from medicinal plants on male fertility. Quinine, a potent alkaloid with strong antimalarial activity is established to possess adverse effects on male and female reproduction through negative feedback inhibition of steroidogenesis [5].

The actions of the extract were reversible as the withdrawal of the purified fractions of *Cnestis ferruginea* led to gradual restoration of the sperm functions to the pre-treatment levels. Although there were still some abnormal sperm and fewer normal sperm in the recovery group, the motility and viability of sperm in the recovery group were almost fully restored. This study therefore showed that the chromatographic fractions of *Cnestis ferruginea* have reversible male antifertility effects. The reproductive effects may be due to quinolizidine alkaloids that were characterized from the purified fractions (F3 and F4) by NMR spectroscopy.

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