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Testicular toxicity of the ethanolic extract of the stems of *Massularia acuminata* (G. Don) Bullock ex Hoyl in rats

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The ethanolic extract of the stems of *Massularia acuminata* is widely used in several regions of Côte d'Ivoire for the improvement of the male sexual performance. The study was carried out with a view to investigate the effect of long term use of the plant on rat. For this purpose, four groups of 6 rats each, aged 8 weeks and weighing on average 125 ± 23.16 g. Groups 2, 3 and 4 were treated repeatedly with the ethanolic extract of the stem of *M. acuminata* at doses of 40, 80 and 160 mg/kg of body weight for 28 days respectively. Group 1, the control group, received only distilled water. In addition to the testicular histological study, body mass and relative testicular mass, sperm motility, concentration and viability were evaluated. This study showed that 40 and 80 mg/kg of ethanolic extract of stems of *M. acuminata* were better tolerated by the testes. However, the 160 mg/kg body weight dose caused a significant reduction in body mass (-4.68 vs +1.30%) and relative testicular mass (1.27 vs 0.45) on day 28 of the experiments. In addition, the result of the study revealed a significant decrease in motility (16.29 vs 41.71%), sperm concentration (10.33 vs 138.83 sperm/µl) and viability compared to the control group. This study showed that at high doses, the ethanolic extract of the stems of *M. acuminata* can disrupt spermatogenesis in rats.

Key words: *Massularia acuminata*, toxicity, rat, testis, spermatozoa.

INTRODUCTION

In all developing countries such as Côte d'Ivoire, the use of medicinal plants is the most common way of solving public health problems, especially in rural areas. According to the World Health Organisation (WHO), more than 80% of the African population uses traditional medicine for their primary health care because of their

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proximity and accessibility (WHO, 2002).

Medicinal plants for aphrodisiac use are increasingly coveted in our society, especially among men, to develop, preserve their sexual capacity or stimulate sexual desire. Among the many aphrodisiac preparations in Côte d'Ivoire, is the ethanolic extract of the stems of *Massularia acuminate*. This preparation is sold in cabarets under the name '4 o'clock in the morning'.

The species *M. acuminata* (G. Don) Bullock ex Hoyl. belonging to the Rubiaceae family, is a plant used in traditional African medicine against various indications, in particular against hernia, sterility, ovarian disorders, fevers, difficult deliveries, rheumatic pains, oral infections, haematuria, poison, dysentery, coughs and especially sexual weaknesses (Aladesanmi et al., 2007; Bouquet, 1969; Kayode and Omotoyinbo, 2008; Neuwinger, 2000; Singh et al., 2011; Yakubu et al., 2008). The phytochemical screening revealed that the ethanolic stem extract of *M. acuminata* contains coumarins, flavonoids flavonoids, tannins, triterpenes, triterpeneaponins, Glycoside, Saponin, Tannin, Flavonoid, Anthocyanin and Anthraquinone sterols (Bankole et al., 2012; Maloueki et al., 2015; Gbogbo et al., 2021).

Pharmacological studies have shown good antibacterial and antifungal activity of ethanolic extracts of *M. acuminata* on Staphylococcus, Streptococcus, Proteus, *Aspergillus favus*, *Candida tropicalis, Candida albican* (Enwa et al., 2016; Olusola et al., 2020).

Other studies have demonstrated the aphrodisiac activity of the roots and stems of *M. acuminata*. These authors observed, in a dose-dependent mode, a significant increase in the frequency of sexual mounts, erections, ejaculations and a decrease in the latency time between two consecutive sexual mounts (Yakubu and Akanji, 2011; Gbogbo et al., 2021). Despite these promising results, there are few data on the effect of its prolonged use on the testes. It is in this context that the present study aims to evaluate the subacute testicular toxicity of the ethanolic extract of *M. acuminata* stems in rats.

MATERIALS AND METHODS

Plant material

The plant material collected in Bonoua, in the department of Grand-Bassam in the South-Comoé region (Côte d'Ivoire), in November 2020. The plant was identified at the National Floristic Centre of the Félix Houphouët Boigny University where a specimen was deposited and identification number (UCJ15291) was collected.

Animals

For this study, male rats *Rattus norgevicus,* aged eight weeks, weighing an average 125 ± 23.16 g were used for the experiment. The rats were bred in the animal house of the Physiology, Pharmacology and Pharmacopoeia Laboratory of the Research Unit of Nangui Abrogoua University. A total of 24 rats were divided into four groups animals were subjected to a temperature of 25 ± 2°C and to an alternation of 12 h of light and 12 h of darkness. The diet consisted of IVOGRAIN® pellets and the rats were provided with tap water. The experimental protocol and animal handling procedures were conducted according to good laboratory practice (OECD, 1998).

Preparation of ethanolic extract

For the preparation of the extract, two hundred grams (200 g) of powder obtained from the stem of *M. acuminata* were macerated in 2.5 L of 96% (v/v) ethanol for 24 h under continuous stirring. The resulting macerate was filtered and then concentrated under reduced pressure at 40°C using a rotary evaporator. The concentrated filtrate was dried in an oven at 40°C. The dry extract obtained constituted the ethanolic extract and was kept for the experiments (Gbogbo et al., 2021).

Toxicity assessment

The subacute toxicity study was determined using OECD guideline 407 (OECD, 2008) which involved the daily oral administration of extracts in increasing doses to four groups of animals, one dose per group for 28 days. The doses of 40, 80 and 160 mg/kg were given to Groups 2, 3 and 4 respectively. Group 1 (control) received only distilled water. Prior to administration of the extracts, the animals in each group were individually marked and weighed. They received a volume of solution of 2 mL/100 g body weight once a day for 28 days by oral gavage using a cannula. The animals were observed individually every morning throughout the study. The influence of the different doses administered was assessed on the basis of the rats' body mass, cytological and histological examinations.

Body mass and relative testicular mass

The body mass of each rat was measured on days D0, D14 and D28. After 28 days of treatment, all rats were anaesthetised and sacrificed by cervical dislocation and the testes were removed and weighed (AVMA, 2020).

Effect of the extract on spermatozoa

Sperm evaluation (sperm motility, concentration and viability)

After the rats were sacrificed, the caudal epididymis of the right testis of each rat was removed, cut into small pieces and transferred to 1 ml of 0.9% sodium chloride solution (0.9%). The mixture was gently shaken to allow the sperm to disperse in the sodium chloride solution, and the sperm suspension was transferred to an Eppendorf tube as described by Talebi et al. (2011). Each sample was then temporarily incubated at 37°C, for 30 min for subsequent sperm analysis.

Sperm motility

Sperm motility was analysed by adding 6 µl of the sperm suspension to the KOVA®-Slide chamber hemocytometer to determine the proportion of motile sperm. The slide was examined under a light microscope at magnification × 400 and motility was recorded in 10 small squares of different grid cells. Sperm showing any degree of movement were considered motile and were counted

Table 1. Evolution of body mass and relative mass of rats.

Groups	Body weight (g)			Relative mass
	D0	D14	D28	Testis
Groupe 1 (Control), Bmg (%)	111.00 ± 29.86	115.00±22.74+ 3.60	116.50±23.91+ 1.3	1.27±0.21
Group 2 (40 mg/kg),Bmg (%)	130.83±23,08	123.66±21, 12- 5.48%*	125.66±17.20+ 1.61	0.94 ± 0.42
Group 3 (80 mg/kg), Bmg (%)	126.33±19.02	128.66±12.21+ 1.84	129.00 ±12.65, 0.26%	0.97 ± 0.53
Group 4 (160 mg/kg), Bmg (%)	129.16±26.28	128.16±28.04 - 0.77*	122.16±28.18, -4.68*	0.45 ± 0.12*

n= 6. D0: day 0; D14: Day 14; D28: Day 28; *: significant decrease ($p < 0.05$) compared to the control; Bmg : Body mass gain.

in groups (motile and immobile). Sperm motility was calculated as follows:

Motility (%) = [Number of motile sperm/total number of sperm] × 100 and expressed as percentage motility (WHO, 1999).

Sperm concentration

Sperm concentration was determined by counting sperm in 10 fields at 400x magnification. For this purpose, 6 µl of the sperm suspension was introduced into the KOVA®-Slide chamber hemocytometer. The sperm concentration was the total average of the sperm in 10 small squares of different grid cells by the following formula:

Sperm count/µl= (Total number of sperm in 10 squares / 10) × 90 (KI, 2021).

Assessment of sperm viability

In a haemolysis tube, 50 µl of sperm suspension was mixed with 50 µl of 0.5% eosin dye. The mixture was shaken for 30 s and then 100 µl of nigrosin is added to the first mixture. A drop of the stained sperm suspension was placed on a slide and covered with a coverslip. Observation is carried out under a light microscope at the x400 objective. Dead spermatozoa appeared purple while live spermatozoa had a blue outline (Dieusaert, 1994). A total of 200 spermatozoa were counted and the proportions of live and dead spermatozoa were calculated.

Histological examinations

At the end of the 28-day experiment, the testes removed and preserved in 10% formalin prior to histological examination on the male reproductive system. The method used was the paraffin embedding technique (Hould, 1984).

Statistical analysis

The statistical study was carried out using the XLSTAT-PRO 7.1 statistical analysis software. The results were analysed using Tukey's and Dunnett's post hoc tests combined with a one-factor Anova. Values are given as the mean followed by the standard error of the mean. Some of the results of the cytological studies were presented as proportions and their analysis was performed using the parametric k-proportion test (G-test). For the study of the relative mass of the organs, we used the following ratio (Yakubu et al., 2008):

Relative mass = (absolute mass of the organ (g)) / (body mass of the animal on the day of sacrifice (g)) × 100.

These tests give us the degree of significance for $p < 0.05$.

RESULTS

Evolution of body mass

The results indicated a loss of body mass in rats treated with ethanolic extract of *M. acuminata* stem at 40 and 160 mg/kg body weight compared to the control group on day 14 of the experiment. A gain in body weight was observed in the group treated with the ethanolic extract at the dose of 80 mg/kg. However, this gain remained below that obtained by the control group (Table 1). On day 28, a gain in body mass was observed in all groups without significant difference except for the rats treated with the 160 mg/kg extract where a loss of mass was observed compared to the control batch.

Relative testicular mass

The results showed a general decrease in the relative mass of the testes in all rats treated with the ethanolic extract of *M. acuminata* stem compared to the mass value observed in the controls. This decrease was particularly significant in rats treated with the 160 mg/kg dose compared to the control (Table 1).

Effect of the extract on spermatozoa

Sperm motility

Assessment of sperm motility did not reveal a significant change in the proportion of motile sperm in rats from the control group compared to those treated with the extract at 40 and 80 mg/kg. In contrast, a significant decrease in the proportion of motile spermatozoa was observed in rats treated with the extract at 160 mg/kg compared to

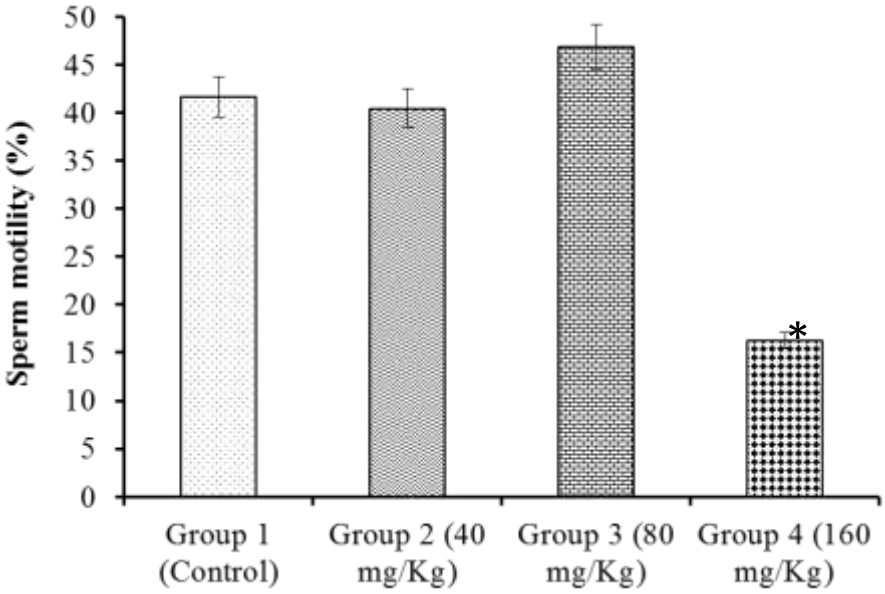

Figure 1. Effect of ethanolic extract of *M. acuminata* on sperm motility in rats.
*: significant decrease compared to the control ($p<0.05$).

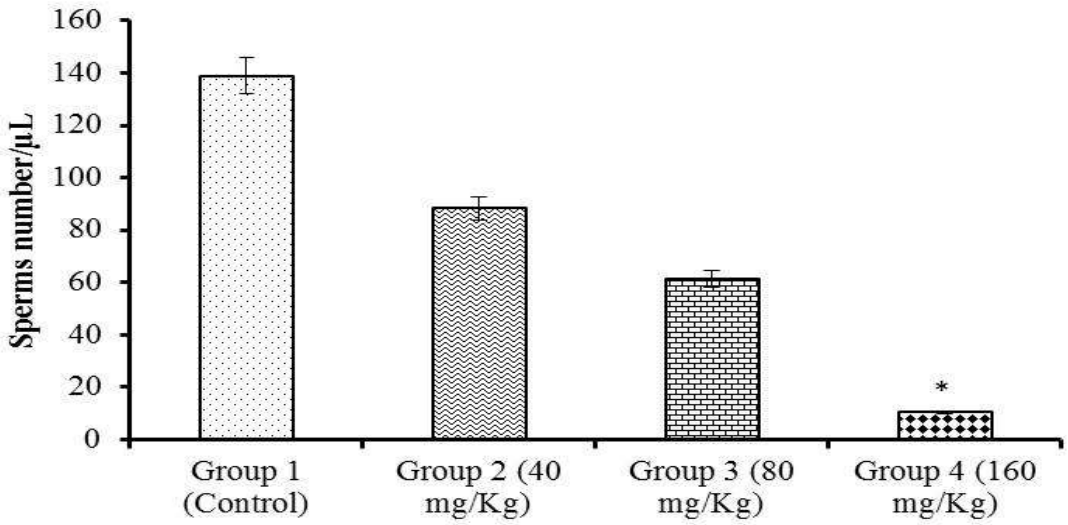

Figure 2. Effect of ethanolic extract of *M. acuminata* on sperm concentration in rats.
*: significant decrease compared to the control ($p<0.05$).

the control group (Figure 1).

Sperm concentration

The results showed (Figure 2) a dose-dependent decrease in sperm concentration in treated rats compared to the control group. Group 4 showed a significantly ($p < 0.05$) lower sperm concentration compared to the control group (10.33 vs. 138.83 sperms/µl).

Sperm viability

The eosin-nigrosin stain was used to observe mainly dead sperm from the rats on the smears. Figure 3A shows dead sperm in a smear from control rats. A decrease in sperm count is observed in micrograph 3B compared to the control. In contrast, a near absence of

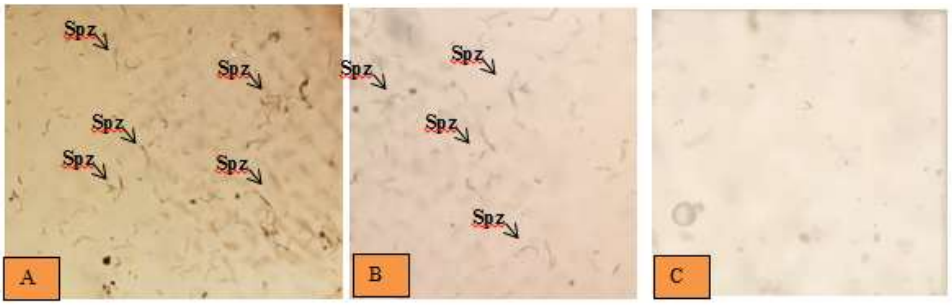

Figure 3. Revealing dead spermatozoa using eosin-nigrosin coloration. A: Field showing dead sperm in a smear from control rats; B: Field showing dead sperm in a smear from rats treated with ethanolic extract at 40 mg/kg; C: Field showing absence of spermatozoa on a smear taken from rats treated with ethanolic extract at 160 mg/kg. Spz: spermatozoa.

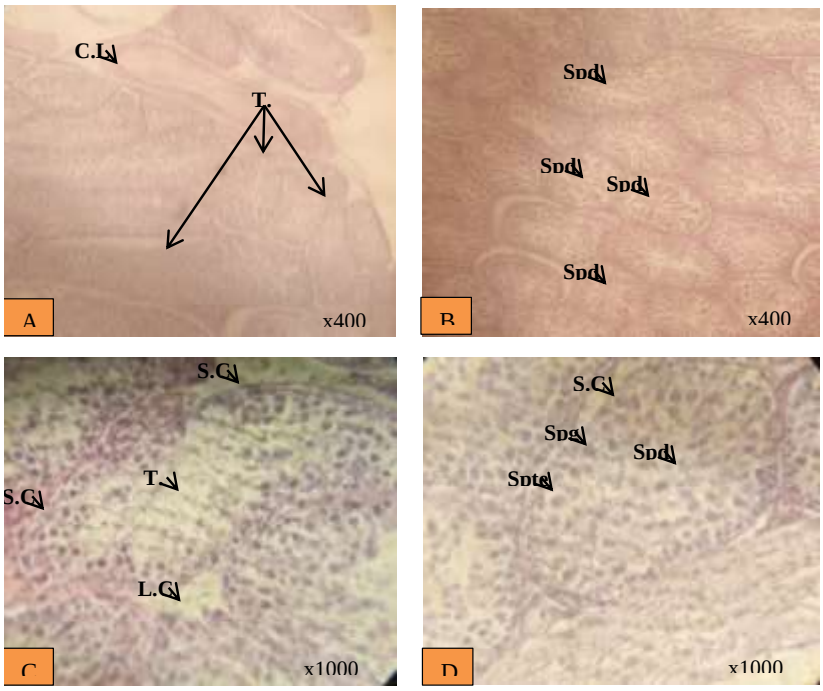

Figure 4. Microphotographs of seminiferous tubules in testicular sections. A and B: Overview of a section from the testes of the control rats, x400. C and D: Cross-section of a x1000 seminiferous tubule from rats treated with ethanolic extract at a dose of 160 mg/kg body weight. Note rarefaction of spermatids and increased thinning of the tubular lumen. Coloration: Eosin-Nigrosin; L.C.: Leydig cell; Spd: Spermatids; S.C.: Sertoli cell; T. Lum: seminiferous tube lumen; Spte : Spermatocytes; Spg: Spermatogonia.

observable spermatozoa was observed in the smears of rats treated with ethanolic extract at a dose of 160 mg/kg (Figure 3C).

Histological study

Histological sections of the testes showed different cells from the different stages of spermatogenesis in the control group, resulting in the presence of spermatids, spermatocytes and spermatogonia (Figure 4A and B). In addition, the microphotographs show Sertoli cells between the germ cells. The lumen of the seminiferous tube is very small. In contrast, histological sections from the group of rats treated with ethanolic extract at 160 mg/kg body weight show disturbances. The disturbances included a rarefaction of spermatids, a greater thinning of the tubular lumen and a disorganisation of the germ cells

in reduced numbers compared to the histological sections in the control rats.

DISCUSSION

This study was conducted to test the effect of prolonged consumption of an ethanolic extract of *M. acuminata* stems in the rats. The results of this study showed that the ethanolic extract of *M. acuminata* stems generally caused a loss of body weight gain in the treated groups compared to the control batch. These results are similar to those obtained by Oloyede et al. (2020) who observed a significant decrease in body mass of mice after oral administration of ethanolic extract of *Lecaniodiscus cupanioides* (Sapindaceae), a plant with aphrodisiac virtue (Olaide and Ajiboye, 2012), at the dose of 100 mg/kg body weight over a period of 30 days.

This decrease in body weight of rats observed in the study could be explained by the presence of tannin and flavonoid in ethanolic extracts of *M. acuminata* (Gbogbo et al., 2021). These authors demonstrated the presence of these secondary metabolites in the ethanolic extract of *M. acuminata*. Indeed, according to some authors, the consumption of plants containing tannins in moderate amounts influences growth (Rochfort et al., 2008; Gutiérrez-Salmeán and Pérez, 2015) and it has been demonstrated that flavonoids can reduce body mass via fatty acid synthesis and thus contribute to the improvement of energy expenditure.

Concerning the testis, this study generally revealed a decrease in their relative mass in all rats treated with ethanolic extract of *M. acuminata* stem compared to the value of the mass observed in controls. These results are similar to those obtained by Dayang and Mahanem (2015) who reported a significant decrease in the relative mass of the testis in rats treated with the 800 mg/kg body weight dose of the methanolic leaves extract of *Andrographis paniculata* (Burm. f.) Wall. Ex (Acanthaceae) in rats. The reduction in relative testicular mass found in this study may be due to a decrease in androgen production. This steroid hormone is responsible for the induction of differentiation and maturation of the male reproductive organs (O'Donnell et al., 2017). Repeated administration of the ethanolic extract of *M. acuminata* stem could therefore inhibit testosterone production by the Leydig cells of the testis, located around and between the seminiferous tubules (Zhou et al., 2019). This study also showed a decrease in sperm motility, concentration and viability, particularly at a dose of 160 mg/kg body weight. These results corroborate those observed in the reduction of testicular mass. A decrease in testosterone production would lead to a disruption of spermatogenesis.

According to studies by Amaral et al. (2014), the decrease in sperm motility is probably due to ATP activity in sperm by uncoupling oxidative phosphorylation from the respiratory chain and preventing the phosphorylation of ADP to ATP, thus rendering sperm immobile. However, motility is considered a key function of good sperm quality as the sperm has to move through the female reproductive tract to fertilise oocyte II. The low sperm concentration and viability of spermatozoa could be due to free radical attacks or oxidative stress. More than any other cell, the spermatozoon is susceptible to free radical damage. The sensitivity of the male gamete to free radical attack is indeed increased by the particular lipid composition of its plasma membrane, rich in polyunsaturated fatty acids, prime targets of reactive oxygen species (Migdal and Serres, 2011). An excessive accumulation of reactive oxygen species can then lead to cell death (Ford, 2004). The administration of ethanolic extract of *M. acuminata* stems would therefore lead to a decline in the number of spermatogonia and their proliferative activity.

With regard to the histology of the rat testicles, the results revealed a disorganised testicular structure with a rarefaction of spermatids, a greater thinning of the tubular lumen and a disorganisation of the germ cells in reduced numbers. These observations confirm the previous results obtained in this study. The administration of 160 mg/kg body weight to rats induces changes in spermatogenesis; the dysfunction of the endocrine system, both pituitary and testicular, is competent enough to influence spermatogenesis negatively (Verhoeven et al., 2010). The toxicity caused by the administration of the ethanolic extract of *M. acuminata* stems would therefore be dose-dependent, as at low doses the effects are less significant than at high doses.

Conclusion

The present study showed that at the high dose of 160 mg/kg body weight, ethanolic extract from the stems of *M. acuminata* could cause a significant decrease in body mass and relative testicular mass of rats compared to the control group. In addition, a decrease in motility, sperm concentration and viability was observed at the same dose. The histological study revealed a disorganised testicular structure with rarefaction of spermatids, increased thinning of the tubular lumen and cellular disorganisation in the seminiferous tubule. Lower doses should be chosen during repeated use of the ethanolic extract of *M. acuminata* stem.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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