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Evaluation of cytogenotoxic potential of *Morinda lucida* leaf extract on Swiss albino male mice using two bioassays

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Abstract:

Background: Utilization of *Morinda lucida* for the treatment of ailments such as malaria, diarrhea, infertility in women, and dysentery in many countries including Nigeria is on increase due to its efficiency, availability, and affordability. However, its cytogenotoxicity has not been elucidated. This study investigated the phytochemical constituents and possible genotoxic and cytotoxic effects of *M. lucida* leaf extract in Swiss albino male mice using bone marrow micronucleus and sperm morphology assays. **Methods:** Plant materials was collected, thoroughly washed, and air-dried at room temperature prior to maceration. The extract was assessed for the presence of the phytochemical compounds. Swiss albino male mice (*Mus musculus*) (n=4) were treated with different concentrations of this extract (400 mg/kg, 800 mg/kg, and 1200 mg/kg) while distilled water was used as negative control. **Results:** Phytochemical analysis revealed that the extract contains saponins, terpenoids, flavonoids, glycosides, and tannins. We observed micronucleated polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) of 3.20%, 1.10%, and 1.95% at different concentrations of 400 mg/kg, 800 mg/kg, and 1200 mg/kg respectively in the treated animals. The result showed no significant increase in the frequency of abnormalities ($p < 0.05$) when compared to the negative control; however, in separate analysis of mono-micronucleated PCE and mono-micronucleated NCE per group, a significant increase at 400 mg/kg and 1200 mg/kg concentrations was observed. The sperm anomalies decreased with increase in concentration. **Conclusions:** This study recommends 800 mg/kg concentration of the plant extract; however, it should be further and properly investigated as it contains some pharmaceutical components that can be used for pharmacological purposes.

Keywords: bone marrow micronucleus, cytogenotoxicity, leaf extract, *Morinda lucida*, phytochemical, sperm morphology

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Introduction

Plants play important roles in the lives of both humans and animals, from providing basic needs such as food, shelter, and clothing to being used as herbal medications. The scientific exploitation of ethno-medicinal herbs for pain relief, wound healing, and abolishing fevers has resulted in the identification of a wide range of compounds that have been developed as new therapies for cancer, hypertension, diabetes, and as anti-infectives. Medicinal plants contain many useful substances some of which are biologically active and are known as secondary metabolites. These substances can be used for therapeutic purposes and have been applied for several decades to serve as precursors for the synthesis of new drugs.

Morinda lucida is a tropical West African tree of medium size about 18–25 m tall with gray to brown bark, white flower, drupe fruit, and yellowish and soft ellipsoid seed [3]. The common name of this plant is brimstone tree; however, it is called different names in different countries. For instance, it is known as Sagogo or Bondoukou alongua in the Ivory Coast; Ewi, Konkroma, or Ewe amake in Ghana; Atak ake or Ewe amake in Togo; and Oruwo in southwestern Nigeria [4]. This plant is among the top widely used herbal plants in most

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African countries including Nigeria, Ghana, and Ivory Coast among others to cure different types of ailments. The leaves are used as “oral teas”, for the traditional treatment of malaria, infertility in women, and as a general febrifuge, analgesic, laxative, and anti-infectious agent because of the antimicrobial active substances [5]. Apart from the leaf extract, the stem bark and root extracts of the plant also contain antimicrobial compounds. In Nigeria, the people of Igbede take the decoction twice or thrice daily as antidiarrheal, while the leaves are used for treatment of infertility in women [6]. Genotoxicity test forms an important part of risk assessment of potential carcinogens. *M. lucida* is an important plant in traditional medicine. Decoctions and infusions or plasters of root, bark, stem, and leaves are recognized remedies against different types of fever, malaria, trypanosomiasis, feverish conditions during child birth, and even cancer. The plant is also employed in cases of diabetes, hypertension, cerebral congestion, dysentery, stomach ache, ulcers, leprosy, and gonorrhea. *M. lucida* is one of the four most used traditional medicines against fever. The administration of an herbal material invariably have its accompanying hazards, precisely the issue of toxicity, mostly due to lack of dosage regimens; thus, the determination of acute toxicity is the primary step in investigating the toxicity of an unknown substance.

In spite of the profound therapeutic advantages possessed by some of the medicinal plants, some constituents of medicinal plants have been shown to be potentially cytogenotoxic, mutagenic, carcinogenic, and teratogenic. Thus, this study aimed at investigating the cytogenotoxic potential of *M. lucida* leaf extract on both somatic and germ cells of Swiss albino male mice using bone marrow micronucleus (MN) and sperm morphology assays, respectively.

Materials and methods

Biological materials

Twenty (20) Swiss albino male mice (*Mus musculus*) of 8–10 weeks old were obtained from the Central Research Laboratory, University of Ilorin, Ilorin, Nigeria. They were acclimatized for 2 weeks in pathogen-free, well-ventilated plastic cages in the animal house of the Department of Zoology, University of Ilorin, Ilorin. During this period, the animals were fed with non-contaminated feed obtained from Aromokeye Pharmacy along Challenge, Ilorin. All through the period of exposure, they were supplied with drinking water on a daily basis, and the beddings were regularly changed, discarded, and disinfected. Throughout the period of the experiment the animals were maintained in the animal house of the Department of Zoology, University of Ilorin, Ilorin.

Collection of plant material

The plant material was collected in August 2017 at Ogundele area of Ilorin west local government, Kwara State. This area has coordinates of 8°28'60"N and 4°27'0"E. The confirmation and authentication of the *M. lucida* leaves was done at the Forest Research Institute of Nigeria, and the herbarium number is FHI 110162.

Extraction of plant material

Morinda lucida leaves were collected, thoroughly washed with water, and air-dried at room temperature to prevent loss of active components present in the plant prior to maceration. About 81.128 g of the macerated leaves was extracted in 810 mL of distilled water and then diluted up to 1000 mL with the solvent vehicle (distilled water) for 24 h with intermittent shaking using a shaker machine. The extract was filtered with muslin cloth and filter paper after which the filtrate extract was concentrated to dryness using a rotatory evaporator to give a concentrated mass of 7.1 g and then kept in the refrigerator at 4 °C until use.

Storage of sample

The plant extract collected was refrigerated at 4 °C until when needed. It was then brought out and diluted to various concentrations at room temperature. The various concentrations were in turn stored in universal bottles and refrigerated at the same temperature throughout the experiment.

Phytochemical tests

The plant extract was assessed for the presence of the phytochemical compounds following standard methods as described by Sofowora et al. [7] and TMS [8].

- i. **Test for tannins:** About 0.4 mL of the plant extract was boiled in 5 mL distilled water. It was filtered, and a few drops of 0.1% FeCl_3 was added to observe for brownish-green or blue-black coloration.
- ii. **Test for saponins:** About 0.4 mL of the sample was boiled in 4 mL distilled water in a water bath and filtered; the filtrate was shaken vigorously for stable persistent froth, which was mixed with three drops olive oil and shaken vigorously to observe for formation of emulsion.
- iii. **Test for flavonoids:** About 0.4 mL sample was heated with 2 mL ethyl acetate over steam bath for 3 min after which the mixture was steeped and 0.4 mL of the filtrate was shaken with 0.1 mL dilute ammonia solution to observe for yellow coloration.
- iv. **Test for steroids:** About 0.4 mL of acetic anhydride was added to 0.1 g of the plant extract and 0.4 mL H_2SO_4 to observe for color change from violet to blue or green.
- v. **Terpenoids (Salkowski test):** About 1 mL of sample was mixed with 0.4 mL chloroform + 0.6 mL concentrated H_2SO_4 carefully to form a layer to observe for reddish-brown coloration.
- vi. **Glycosides (Keller-Killani test):** About 0.5 mL of the sample was treated with 0.2 mL of glacial acetic acid containing one drop of FeCl_3 solution and that had also been underlaid with 1 mL concentrated H_2SO_4 to observe for brown ring and/or violet ring below the brown ring or green ring through the thin layer.
- vii. **Test for alkaloids:** About 10 mL of water was added to the sample, and to 1 mL of this, two drops of Wagner's reagent (iodo-potassium iodide, obtained by dissolving 0.2 g of iodine and 0.6 g of KI in 1 mL of water) to observe for brown or reddish-brown precipitate.

Experimental design

The bone marrow MN test was used to assess the somatic genotoxicity of the extract. For the genotoxicity assessment, four groups of mice ($n = 4$) with average weight of 20–25 g per extract concentration were used. The groups were divided and named as A, B, C, and D. Each mouse per group was orally administered with different concentration of the extracts per day for 5 consecutive days (24 h interval). Two mice ($n = 2$) per group were sacrificed after the last exposure for bone marrow MN assay and at 35 days after the last exposure for sperm morphology assays. The 35 days is necessary in order to allow spermatogenesis in male mice to be completed before sacrificing the animals since one mouse spermatogenic cycle from spermatogonia to mature spermatozoa takes 34.5 days as described by Wyrobek [9] and Lamming [10].

Group A (negative control) received 0.2 mL of distilled water orally for 5 consecutive days.

Group B (400 mg/kg) received 0.2 mL of *M. lucida* orally for 5 consecutive days.

Group C (800 mg/kg) received 0.2 mL of *M. lucida* orally for 5 consecutive days.

Group D (1200 mg/kg) received 0.2 mL of *M. lucida* orally for 5 consecutive days.

These concentrations were selected based on previous research by Yinusa et al. [11], and all experiments were done in two replicates.

The bone marrow preparation for MN assessment was carried out according to Schmid [12] and Alabi and Bakare [13]. During the period of treatment, the mice were fed and had unlimited access to water. After the period of treatment, the mice ($n = 2$) were sacrificed by cervical dislocation. The femur of each mouse was surgically removed and flushed from the bones with fetal bovine serum (FBS). It was centrifuged at 2000 rpm for 5 min, and slides were stained with May-Grunwald and Giemsa stains. At least 2000 cells per mouse were scored at $\times 1000$ for MN in polychromatic erythrocytes (MNPCE) and normochromatic erythrocytes (MNNCE).

Cell suspension and scoring

Two mice ($n = 2$) from each group were sacrificed by cervical dislocation; the femurs were removed and freed from muscles using wool gauze. The distal and proximal epiphysis of each femur was cut circumspectly to make a hole for the aspiration but not leaving the diaphysis short. About 1 mL of FBS in an insulin syringe was inserted at one end of the hole to flush the bone marrow inside a centrifuge tube and was properly mixed. The content was then centrifuged at 2000 rpm for 5 min, and the supernatant was discarded. The pellet was taken by micropipette and mixed with 1 mL of FBS. It was thoroughly shaken and centrifuged again at 2000 rpm for 5 min. The supernatant was discarded. The pellet was mixed with 0.5 mL of FBS and mixed gently for slides preparation, and a drop of the viscous cell suspension was taken using the micropipette. The slides were air-dried for 24 h, stained afterwards in 0.4 mL of May-Grunwald for 5 min, and later transferred into 1:1 (v/v) distilled water and May-Grunwald stain for another 5 min. The slides were removed and rinsed in distilled water, air-dried, and further stained in 5% Giemsa for 5 min. After 5 min, the slides were rinsed with distilled water and air-dried. Subsequently, the slides were dipped in xylene to make a clear film and mount with cover slip using 2 drops of dibutylphthalate polystyrene xylene (DPX).

For each suspension, the slides were examined at 1000-fold magnification with oil immersion objective; a total of 2000 of cells were thus examined per group. The incidence of micronucleated PCEs (MNPCEs) and NCEs (MNNCEs) per 2000 cells (PCEs + NCEs) per animal were scored and compared with the negative control to give the genotoxic end point.

Sperm morphology assay

Induction of sperm abnormalities was done in accordance with standard procedures with slight modification according to the method described by Wyrobek et al. [9] and Bakare et al. [14]. Briefly, two mice from each group were carefully sacrificed by cervical dislocation, and their caudal epididymis were surgically removed. Sperm suspensions were prepared from each group by mincing the caudal epididymis in buffered physiological saline and pipetting the resulting suspension. A fraction of each suspension was then mixed (9:1) with normal saline and 1% eosin Y, respectively, for 45 min. For each suspension, 1000 sperm cells were examined at 1000-fold magnification (oil immersion) using an Olympus microscope; thus, a total of 2000 sperm cells were scored for sperm abnormalities.

Percentage abnormality of micronucleated PCEs for each concentration treatment for both the test and control groups were calculated using the formula below:

$$\text{Abnormality \%} = \frac{\text{Number of abnormal PCEs counted} \times 100}{\text{Total number of erythrocytes counted}}$$

While the percentage abnormality of spermatozoa for each concentration treatment for both the test and control groups were calculated using the formula below:

$$\text{Abnormality \%} = \frac{\text{Number of abnormal sperm counted} \times 100}{\text{Total number of sperm cells counted}}$$

Statistical analysis

The Statistical Package for Social Sciences 21.0 was applied to evaluate the distribution of micronucleated PCEs and abnormal spermatozoa. The difference between the negative control and the individual concentration groups were analyzed using one-way ANOVA at the $p < 0.05$ level. Duncan's new multiple range test was used to compare the difference between the experimental groups and the control group. The mean and standard error were calculated and used to present the results for each group.

Results

Phytochemical screening result

The phytochemical constituents of the stock sample of the plant extract (*M. lucida* leaf) indicates the presence of saponin, terpenoids, flavonoids, glycosides, and tannins. However, alkaloids and steroids were not detected from the plant extract

Bone marrow micronucleus assay

Measurement of both PCE and NCE were made in both control and treated mice on the fifth day. There were 3.20%, 1.10%, and 1.95% micronucleated PCE and NCE at the tested concentrations of 400 mg/kg, 800 mg/kg, and 1200 mg/kg, respectively (Table 1). However, there was no significant difference in the frequency of abnormalities in all the tested concentrations when compared to the negative control (Table 1). Table 2 shows the frequency of MNPCE and MNNCE of different concentration groups. Therein, a significant increase ($p < 0.05$) was observed at 400 mg/kg body weight. The frequency of MNPCE observed in mice after 5 days of exposure to different concentrations of *M. lucida* was higher at 400 mg/kg body weight when compared to the other concentrations as well as the negative control (Figure 1). However, the frequency of MNPCE and MNNCE at different concentrations of *M. lucida* leaf extracts are shown in Figure 2. Figure 3 shows the photomicrograph of normal PCE, NCE, MNPCE, MNNCE, and bi-micronucleated.

Table 1: Frequency of MNPCE observed in the bone marrow of mice exposed to different concentrations of *M. lucida* extract after 5 days of exposure.

Concentration levels, mg/kg	Total number of abnormalities	% of abnormalities	Mean $\pm$ SE
Negative control	21	1.05	10.5 $\pm$ 0.00
400 mg/kg	64	3.20	32.25 $\pm$ 16.75
800 mg/kg	22	1.10	11 $\pm$ 2.00
1200 mg/kg	39	1.95	19.5 $\pm$ 0.00

Negative control = distilled water; number of animals/group = 4; total number of PCE and NCE scored/concentration = 2000 erythrocytes; significance level = 0.05.

Table 2: Frequency of MNPCE and MNNCE at different concentration groups.

Concentration	MNPCE	MNNCE	MNPCE, mean $\pm$ SE	MNNCE, mean $\pm$ SE
Negative control	21	0	10.5 $\pm$ 2.5	0.0 $\pm$ 0.0
400 mg/kg	59	5	29.5 $\pm$ 5.5 ^a	2.5 $\pm$ 0.71 ^a
800 mg/kg	20	2	10.0 $\pm$ 1.0	1.0 $\pm$ 0.0
1200 mg/kg	38	1	19.0 $\pm$ 3.0 ^a	0.5 $\pm$ 0.71

Negative control = distilled water. ^aSignificant at $p < 0.05$.

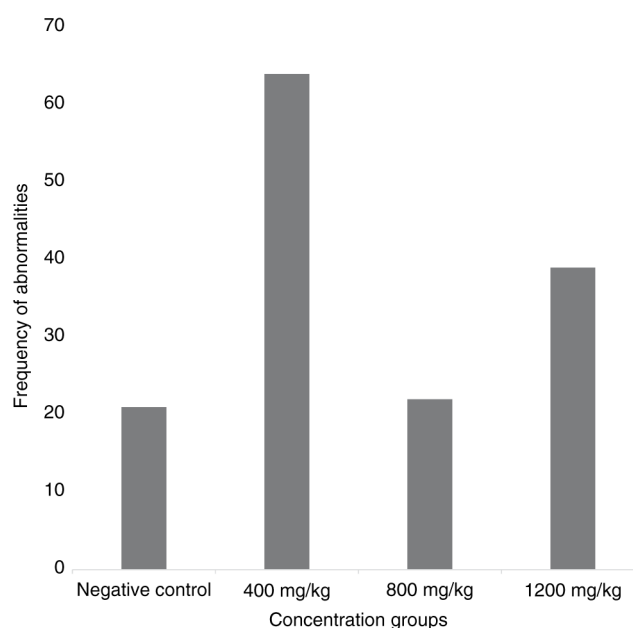


Figure 1: Frequency of MNPCE observed in mice after 5 days of exposure to different concentrations of *M. lucida*. Negative control = distilled water.

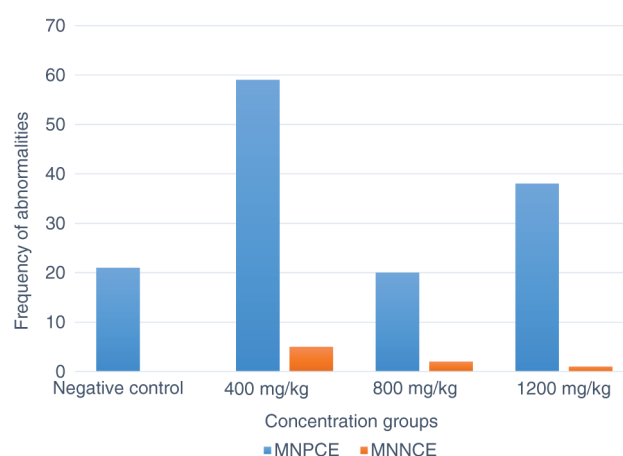


Figure 2: Frequency of MNPCE and MNNCE observed in mice exposed to different concentration of *M. lucida* leaf extracts. Negative control = distilled water.

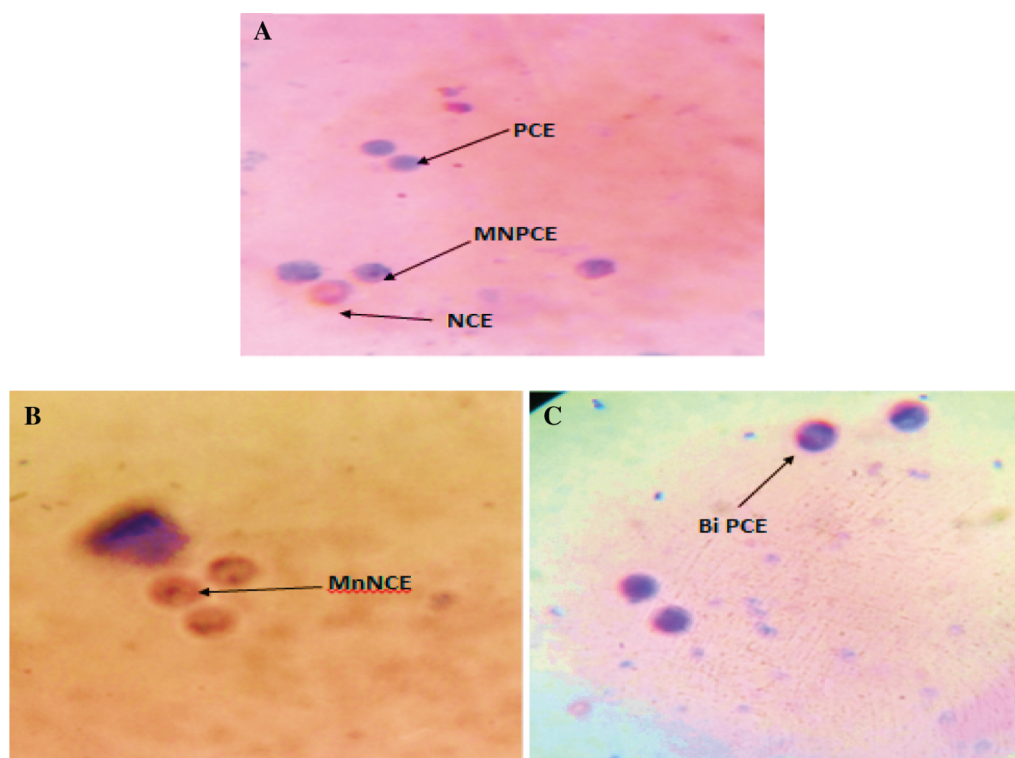


Figure 3: Micronuclei abnormalities in the bone marrow of mice exposed to different concentrations of *M. lucida* leaf extract.

(A) PCE, polychromatic erythrocytes; NCE, normochromatic erythrocyte; MNPCE, mono-micronucleated polychromatic erythrocyte; (B) MNCE, mono-micronucleated normochromatic erythrocyte; (C) BiPCE, bi-micronucleated polychromatic erythrocyte (X1000).

Sperm head abnormality test

The summary of morphologically abnormal sperm induced in Swiss albino male mice by different concentrations of *M. lucida* is presented in Table 3. At the lowest concentration (400 mg/kg), 1.43% of the sperms scored were abnormal in shape. It was also observed that the percentage abnormality decreased with increase in concentration administered at first in 400 mg/kg (1.43%) and 800 mg/kg (1.26%) but decreased at 1200 mg/kg (0.93%). The abnormalities observed in the sperm were amorphous head, coiled tail, double heads, hook at wrong angle, wrong tail attachment, folded sperm, banana shaped, and kinky tail. Amorphous head was the most common abnormality observed in the sperm.

Table 3: Summary of sperm abnormality types and frequency in Swiss albino mice after 5 days of *M. lucida* administration.

Conc./Abnormalities	AH	CT	DH	KT	HWA	WTA	FS	BS	Total	Abnormality (%)	X ± SE
Negative control	2	0	0	0	0	1	1	0	4	0.20	1.333 ± 0.3333
400 mg/kg	10	6	4	4	0	9	5	5	43	2.15	0.000 ± 4.9329
800 mg/kg	5	2	8	0	5	10	0	8	38	1.90	0.666 ± 2.6666
1200 mg/kg	4	0	0	12	0	0	3	9	28	1.40	0.333 ± 2.9627

AH, amorphous head; CT, coiled tail; DH, double head; KT, kinky tail; HWA, hook at wrong angle; WTA, wrong tail attachment; FS, folded sperm; BS, banana shape. Negative control = distilled water.

Discussion

Herbal medicines have become a popular form of therapy in both developed and developing countries. They are believed to be non-toxic, with little or no side effects compared to conventional drugs. Despite their widespread recognition, herbal medicines could cause toxicity due to their inherent properties, contamination, and variability of active or toxic components because of variations in culturing, processing, and preparation techniques [15]. In this study, the cytogenotoxicity of *M. lucida* leaf extract in Swiss albino male mice using bone marrow MN and sperm morphology assays was investigated. The evaluation of toxic, cytotoxic, and genotoxic damage caused by plant compounds is of fundamental importance in minimizing the possible risks of these agents, especially when they are part of long-term treatment [16]. Our result showed that with three different concentrations of *M. lucida* leaf extract there was no significant difference ($p < 0.05$) in the frequency of abnormalities at all the tested concentrations when compared to the negative control. Although there was a significant difference ($p < 0.05$) in the frequency of micronucleated PCEs and NCEs at concentrations of 400 mg/kg and 1200 mg/kg ($p < 0.05$), the analysis ran on the micronucleated NCEs of individual doses of the extract showed a significant increase ($p < 0.05$) in the 400 mg/kg group. This study is not in alignment with a study carried out by Adejo and Atawodi [17] wherein the methanolic extracts of the leaf was genotoxic at all the doses, although their study used plasmid nicking assay for their genotoxicity testing, while this study utilized bone marrow MN assay which detects clastogenicity due to chromosome breakage and aneugenicity due to chromosome lagging resulting from dysfunctioning of the mitotic apparatus, and micronuclei appear in cells due to chromosomal damage during the last mitosis [18]. In a separate experiment carried out by Ogunlana [19], the antioxidant activity of *M. lucida* (Benth.) stem bark investigated showed that the methanolic extract of stem bark of *M. lucida* exhibits antioxidant activity, and thus, the total phenolic content, antioxidant activity, and reducing power of the extract had a direct relationship with the observed activity.

The induction of sperm abnormalities by any agent is of great significance. This has led to the advent of sperm morphology assay. This assay is now widely used as short-term assays for the detection of agents which are capable of altering spermatogenesis and has been shown to have good predictive potential for the identification of germ cell mutagens [20]. It has the potential of allowing identification of chemicals that induce spermatogenic dysfunction and perhaps heritable mutation [9]. In relation to mutagenicity, sperm shape has been reported to be genetically controlled by numerous genes [21]. Many antimalarial agents have been demonstrated to have various degrees of antifertility activities [22]. The use of *M. lucida* extract in the treatment of various ailments is increasing, but its impacts on the reproductive system have not yet been elucidated. The present study also investigated the cytotoxic potential of *M. lucida* leaf extract on reproductive cells of Swiss albino male mice. Our results showed that the sperm anomalies decreased with increase in concentrations. However, these anomalies (folded sperm, amorphous, banana shape, etc.) at all concentrations showed no statistical significance ($p < 0.05$) when compared to the negative control. This result is in line with a study conducted by Raji et al. [23] wherein methanolic extracts of *M. lucida* showed a significant increase ($p < 0.05$) in sperm abnormalities at 400 mg/kg for 4 and 13 weeks period of exposure. In a separate study, male rats treated with 400 mg/kg of *M. lucida* for 4 weeks and 13 weeks to evaluate the antispermatic effect of the extract revealed degeneration and disorganization of the plasmalemma in the basal portion of some of the seminiferous tubules [11]. Although the mechanisms responsible for nuclear aberration have not been fully explained, these abnormalities are considered to be indicators of cytotoxic damage and, therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys [24].

Although the phytochemical screening of the *M. lucida* leaves extract showed the presence of saponins, terpenoids, flavonoids, glycosides, and tannins, taking of low dose or overdose of this plant extract might be responsible for the abnormalities observed at 400 mg/kg and 1200 mg/kg compared to the intermediate concentration (800 mg/kg) of the treated mice. According to Adeleye et al. [25], the benefits derived from *M. lucida* is due to the high contents of vitamins A, K, and E, alkaloids, and other phytochemicals such as flavonoids and phenolic steroids it contains. Flavonoids are effective as free radical scavengers and have anti-allergic, anti-inflammatory, antiviral, anti-proliferative, and anti-carcinogenic properties. It is capable of improving semen quality in infertile men with oligospermia [26]. Saponins are naturally occurring surface-active glycosides found in many plants and helps in reducing the concentration of plasma cholesterol and regulating the immune systems [27]. Tannins have traditionally been reported to have antinutritional properties depending upon their chemical structure and dosage, while terpenoids have the tendency to regulate metabolic processes [25]. The glycosides containing anthraquinone aglycone group can be used to treat and prevent constipation [25].

Conclusions

Our study revealed that oral administration of *M. lucida* leaf extract at various concentrations (400 mg/kg, 800 mg/kg, and 1200 mg/kg) had an insignificant decrease in the inductions of MNPCE, MNNE, and sperm cells in the tested mice when compared to the negative control and hence it is not cytogenotoxic. The antigenotoxicity properties of this plant can be attributed to the presence of flavonoids, tannins, and saponins among others. Thus, the 800 mg/kg concentration of *M. lucida* can be recommended for the treatment of various diseases as it induces an insignificant level of abnormalities compared to that of 400 mg/kg (lowest) and 1200 mg/kg (highest) concentrations. Due to paucity of data, further studies are recommended to be carried out on *M. lucida* extract from its leaf, stem, and root to elucidate its mechanism.

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Competing interests: Authors state no conflict of interest.

Ethical approval: This study was carried out following strictly the guidelines on care and use of laboratory animals of the Ethical Committee of the University of Ilorin, Ilorin, Nigeria.

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