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Research Article

Demonstration of Sperm Morphology in Mice and Lizard Using Aqueous Extracts of *Lawsonia inermis* Leaf*, Bougainvillea spectabilis* Flower and *Hibiscus sabdariffa* Calyx

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ABSTRACT

Spermatozoon is a mature male reproductive cell that is formed in the male reproductive system through a process called spermatogenesis. Available stains and techniques for sperm morphology are currently expensive with sophisticated procedures and synthetic dyes. The study aimed to demonstrate spermatozoa morphology in mice and lizards using aqueous extracts of *Lawsonia Inermis* leaf (LIL)*, Bougainvillea spectabilis* flower (BSF), *a*nd *Hibiscus sabdariffa* calyx (HSC). The epididymal spermatozoa of mice and lizards were smeared on a glass slide, fixed, and stained with crystal violet, LIL, BSF, and HSC. Crystal violet showed complete staining of mice and lizard spermatozoa demonstrating both head and tail morphology. LIL, BSF, and HSC also showed complete staining of the tail in both animals. However, only the outline of the head was stained. Counterstaining the mice spermatozoa with eosin and haematoxylin produced a pink-stained and grey-stained head respectively. Counterstaining of lizard spermatozoa with eosin and haematoxylin did not improve head staining. In conclusion, the aqueous extract LIL, BSF, and HSC demonstrated both head and tail morphology of the spermatozoa in mice when counterstained with eosin. However, BSF and HSC stains did not show the head morphology in lizards even with counterstaining.

Keywords: *Bougainvillea spectabilis; Hibiscus sabdariffa; Lawsonia Inermis;* Morphology; Spermatozoa

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INTRODUCTION

Spermatozoon is a mature male reproductive cell formed in the male reproductive system through spermatogenesis; which involves: spermatogonia, meiosis, and spermiogenesis (Adeniji, *et al.,* 2010; Jan *et al.,* 2012). The fully developed spermatozoon is an elongated cell with an oval head and long slender tail called a flagellum (Adeniji *et al.,* 2010). Spermatogenesis is the process of male germ cell differentiation to spermatozoa. It begins with the differentiation of undifferentiated spermatogonial cells. The differentiated spermatogonia enter meiosis, the most important part of spermatogenesis. Germ cells, now called spermatocytes, undergo meiotic recombination and reductive cell division, which results in spermatids (Kulibin and Malolina, 2023). Finally, round spermatids are transformed into elongated spermatids and spermatozoa through spermiogenesis (Linn *et al.,* 2021; Kulibin and Malolina, 2023). Normal spermatogenesis relies on Sertoli cells, which preserve cell junctions while providing nutrients for mitosis and meiosis of male germ cells (Linn *et al.,* 2021). About 25% or more of abnormal sperm leads to reduced fertility (Gyeongsang National University, 2005). The primary function of a spermatozoon is to deliver genetic material (DNA) to the female ovum during fertilization by penetrating the outer layer of the ovum and fusing with it (Adeniji, *et al.,* 2010).

Assessment of sperm morphology is the most important criterion for determining the quality of a semen sample (Lingappa *et al.,* 2015). The assessments should be performed by trained personnel, familiar with all principles to designate spermatozoa as abnormal (Pelzman and Sandlow, 2024**).** The goal of identifying an ideal technique for sperm staining is to ease the visualization of cells and identify abnormal cells (Lingappa *et al.,* 2015). Many synthetic dyes such as haematoxylin & Eosin, Giemsa, Papanicolau, Eosin-Nigrosin, and Leishmann have been used for spermatozoa staining (Lingappa *et al.,* 2015). Most stains currently used are chemically synthesized from petroleum byproducts and are hazardous to human health (Ratna and Padhi, 2012). Natural vegetable dyes now gain prominence globally, especially in developing countries due to their eco-friendly nature, and hence, their promising usage was used as histopathological stains (Akinloye *et al*., 2009). *Lawsonia inermis,* also known as Henna, is a shrub in the Lythraceae family. It is primarily used in cosmetics as a pigment for colouring hair and nails, imparting a red-yellow tint (Ihuma *et al.,* 2012). Henna leaves are used as a prophylactic against skin diseases and in treating boils, burns, and bruises (Varghese *et al.,* 2011). *Bougainvillea spectabilis* Wild is a woody vine species belonging to the Nyctaginaceae family and was first discovered by a French navigator, Louis Antoine Bougainville in Brazil in 1786 (Kobayashi*, et al.,* 2007). Bougainvillea spectabilis has been used to treat various ailments such as respiratory infections, gastrointestinal disorders, and skin conditions (Al-Snafi, 2015). *Hibiscus sabdariffa* is a plant grown in many African and Asian countries. It produced pigments that can produce dye after some natural processing (Mohammed *et al.,* 2012). The study aimed to demonstrate spermatozoa morphology in mice and lizards using aqueous extracts of *Lawsonia Inermis* leaf (LIL)*, Bougainvillea spectabilis* flower (BSF), and *Hibiscus sabdariffa* calyx (HSC).

MATERIALS AND METHODS

Plant Authentication and Extraction

Lawsonia Inermis leaf (LIL)*, Bougainvillea spectabilis* flower (BSF), and *Hibiscus sabdariffa* calyx (HSC) were authenticated at the Faculty of Pharmacy Herbarium, University of Maiduguri with the following specimen numbers UMM/FPH/LYR/001, UMM/FPH/NYG/001, and UMM/FPH/MAV/002 respectively. LIL, BSF, and HSC were pulverised, and each was dissolved in distilled water (486g in 10 litres) at 5-8 ^{o}C for two days. It was filtered and the filtrate evaporated to dryness in an oven at 45 ^{o}C.

Ethical Consideration

The Ethical Committee, Department of Human Anatomy at the University of Maiduguri approved the research (UM/HA/UGP23.24-005). It was conducted following the National Institute of Health Guide for the Care and Use of Laboratory Animals. To minimize suffering, the mice and lizards were anesthetized with ketamine injections before collecting the epididymides.

Experimental Animal

The study used two Balb/c male mice (10 weeks old) and two male redhead *Agama agama* lizards. The mice were purchased from the Department of Biochemistry animal house while the lizards were caught from the bush near the gross anatomy laboratory at the University of Maiduguri Campus.

Sperm Procurement and Staining

The mice and lizards were anaesthetised with ketamine injection, and the epididymides were dissected and teased in normal saline. Thirty smears were made on glass slides for each epididymis, the smears were fixed in methanol and stained with crystal violet (CV), LIL, BSF, and HSC for five, ten, and fifteen minutes. Some slides stained with LIL, BSF, and HSC were counter-stained with haematoxylin, and others with eosin. The staining solution consists of 2 g of each extract in 10 ml of distilled water. The stains were washed in running water, air-dried, and mounted with Dibutylphthalate Polystyrene Xylene (DPX). The slides were observed with a light microscope and micrographs were taken with a microscope camera at x400 magnification (Dibal *et al*., 2020).

RESULTS

Table 1 shows the staining capacity of LIL, BSF, and HSC in mice spermatozoa. The spermatozoa of mice stained with CV showed complete staining of the head (blue) and tail (black). However, staining with LIL, BSF, and HSC showed a complete staining of the tail (black) with an outline of the head. Counterstaining with eosin resulted in a pink-stained head with a black outline while counterstaining with haematoxylin showed a grey-stained head (Plates 1, 2 & 3). Table 2 shows the staining capacity of LIL, BSF, and HSC in lizard spermatozoa. The spermatozoa of the lizard stained with CV showed complete staining of the head (blue) and tail (black). Staining with LIL also demonstrated complete staining of the head (grey) and tail (black). Counterstaining the LIL-stained spermatozoa with eosin showed a pink-stained head. The BSF and HSC-stained spermatozoa demonstrated only the tail morphology. Furthermore, counterstaining with eosin and haematoxylin did not improve the head staining (Plates 4, 5, & 6).

Table 1: Staining capacity of aqueous extracts of *Lawsonia inermis* leaf, *Bougainvillea spectabilis* flower, *Hibiscus sabdariffa* calyx on mice spermatozoa.

Stains	Preferential binding with parts of spermatozoa	
	Head	**Tail**
Crystal violet	Complete (blue)	Complete (black)
Lawsonia inermis	Outline	Complete (black)
Lawsonia inermis and eosin	Complete (pink)	Complete (black)
Lawsonia inermis and haematoxylin	Complete (grey)	Complete (black)
Bougainvillea spectabilis	Outline	Complete (black)
Bougainvillea spectabilis and eosin	Complete (pink)	Complete (black)
Bougainvillea spectabilis and haematoxylin	Complete (grey)	Complete (black)
Hibiscus sabdariffa	Outline	Complete (black)
Hibiscus sabdariffa and eosin	Complete (grey)	Complete (black)
Hibiscus sabdariffa and haematoxylin	Complete (pink)	Complete (black)

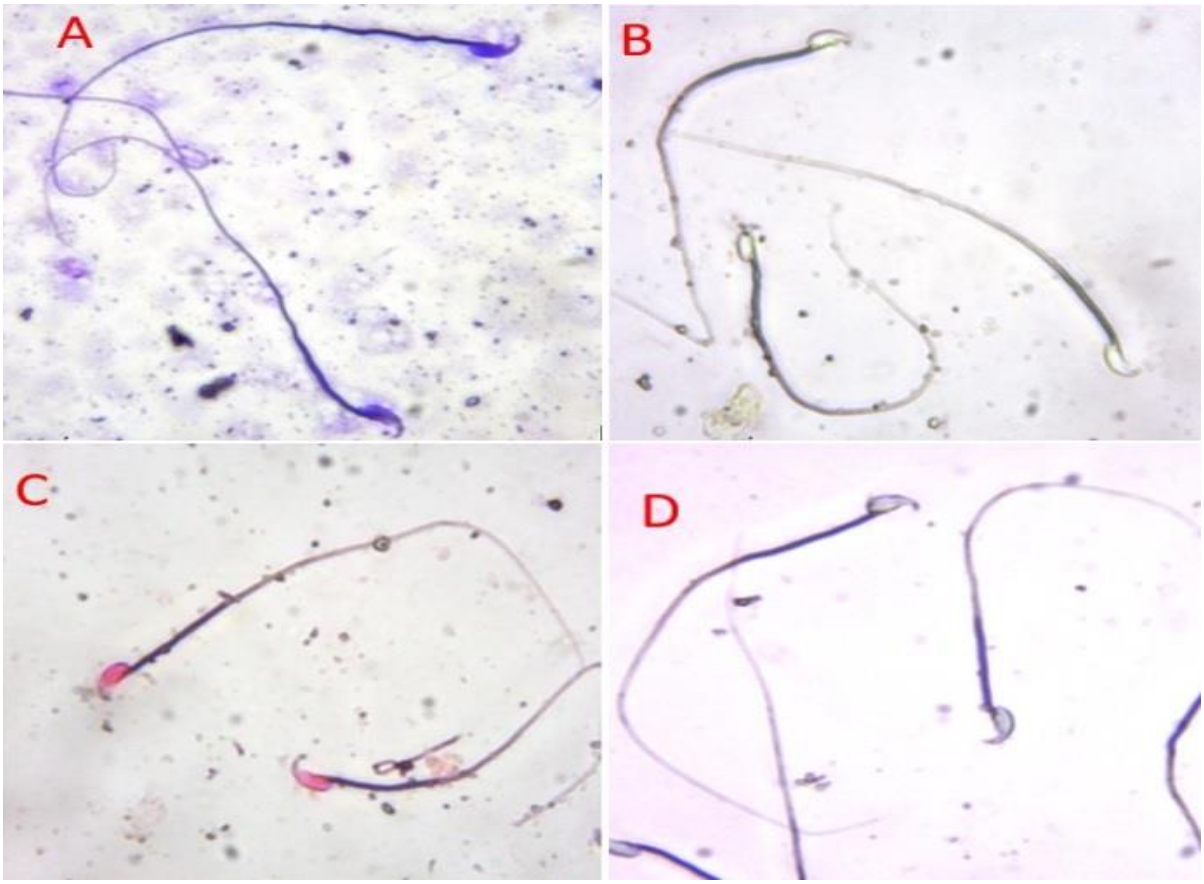

Plate 1: Photomicrograph of mice spermatozoa stained with aqueous extract of *Lawsonia inermis* leaf showing complete staining of the head in A, C & D with an outline of the head in C. A= Crystal violet stain, B= *Lawsonia inermis*, C= *Lawsonia inermis* counter-stained with eosin, D= *Lawsonia inermis* counter-stained with haematoxylin, x400 magnification

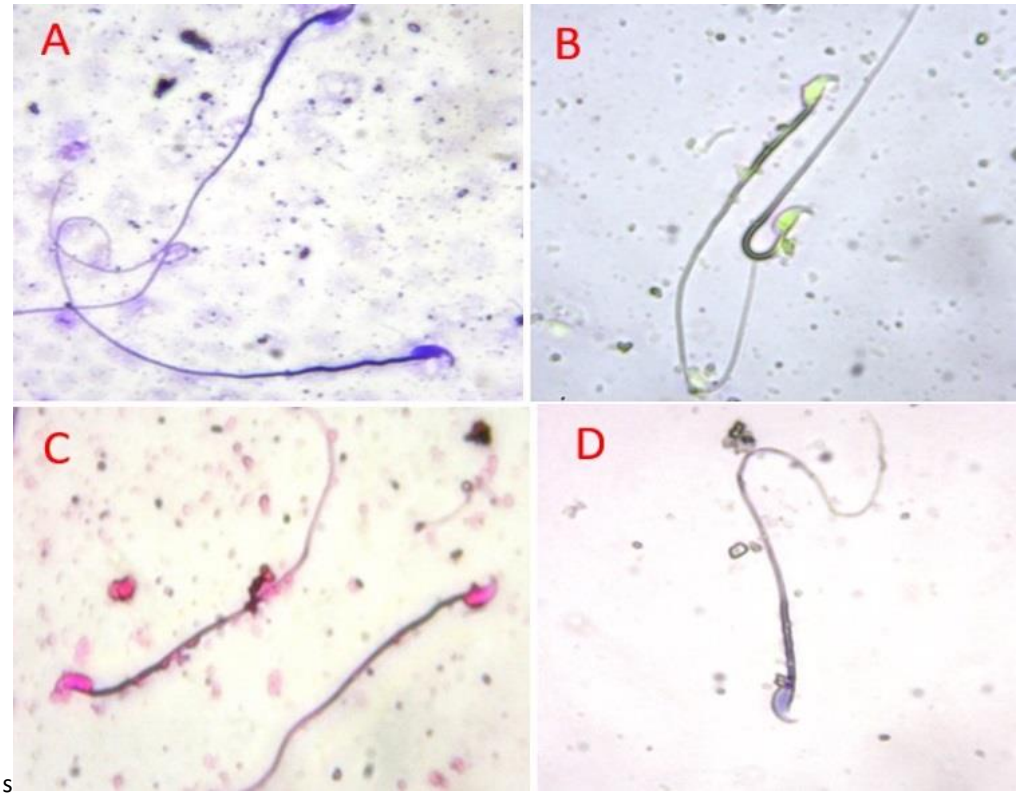

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Plate 2: Photomicrograph of mice spermatozoa stained with *Bougainvillea spectabilis* flower showing complete staining of the head and tail in A, C, & D. C showed an outline of the head and complete staining of the tail. A= Crystal violet stain, B= *Bougainvillaea spectabilis*, C= *Bougainvillea spectabilis* counter-stained with eosin, D= *Bougainvillaea spectabilis* counter-stained with haematoxylin, x400 magnification

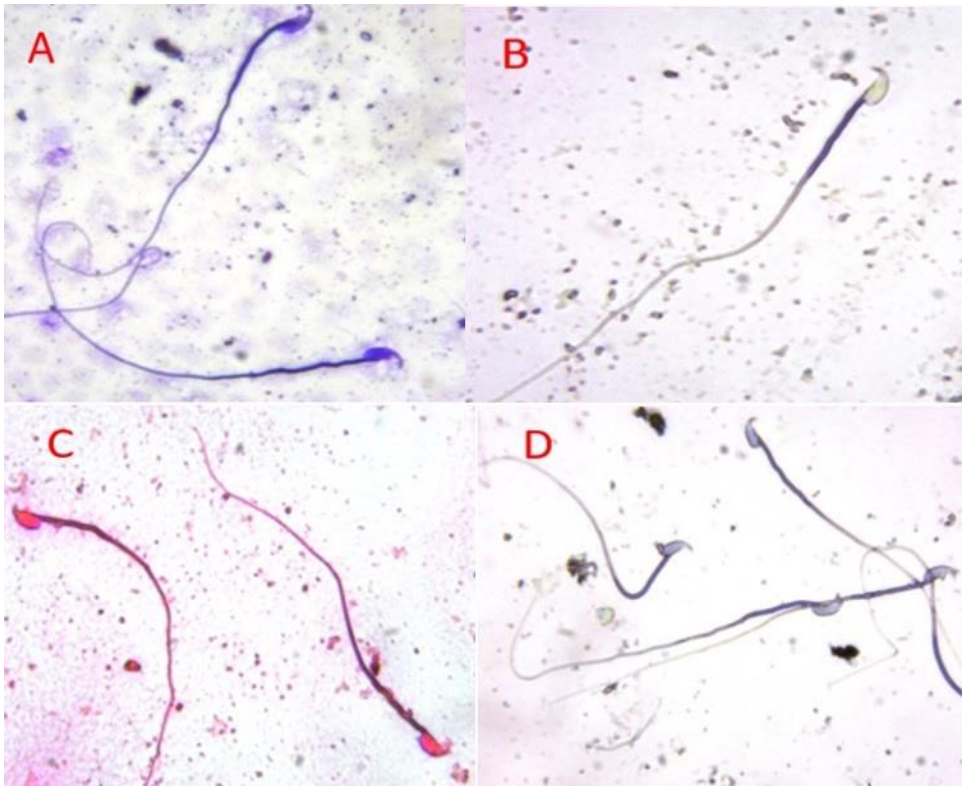

Plate 3: Photomicrograph of mice spermatozoa stained with *Hibiscus sabdariffa* calyx showing complete staining of the head and tail in A, C, & D with an outline of the head in C. A= Crystal violet stain, B= *Hibiscus*

***sabdariffa*, C= *Hibiscus sabdariffa* counter-stained with eosin, D= *Hibiscus sabdariffa* counter-stained with haematoxylin, x400 magnification**

Table 2: Staining capacity of aqueous extract of *Lawsonia inermis* leaf, *Bougainvillea spectabilis* flower, *Hibiscus sabdariffa* calyx on lizard spermatozoa.

Stains	Preferential binding with parts of spermatozoa	
	Head	**Tail**
Crystal violet	Complete (blue)	Complete (black)
Lawsonia inermis	Complete (grey)	Complete (black)
Lawsonia inermis and eosin	Complete (pink)	Complete (black)
Lawsonia inermis and haematoxylin	Outline	Complete (black)
Bougainvillea spectabilis	No staining	Complete (black)
Bougainvillea spectabilis and eosin	No staining	Complete (black)
Bougainvillea spectabilis and haematoxylin	No staining	Complete (black)
Hibiscus sabdariffa	No staining	Complete (black)
Hibiscus sabdariffa and eosin	No staining	Complete (black)
Hibiscus sabdariffa and haematoxylin	Outline	Complete (black)

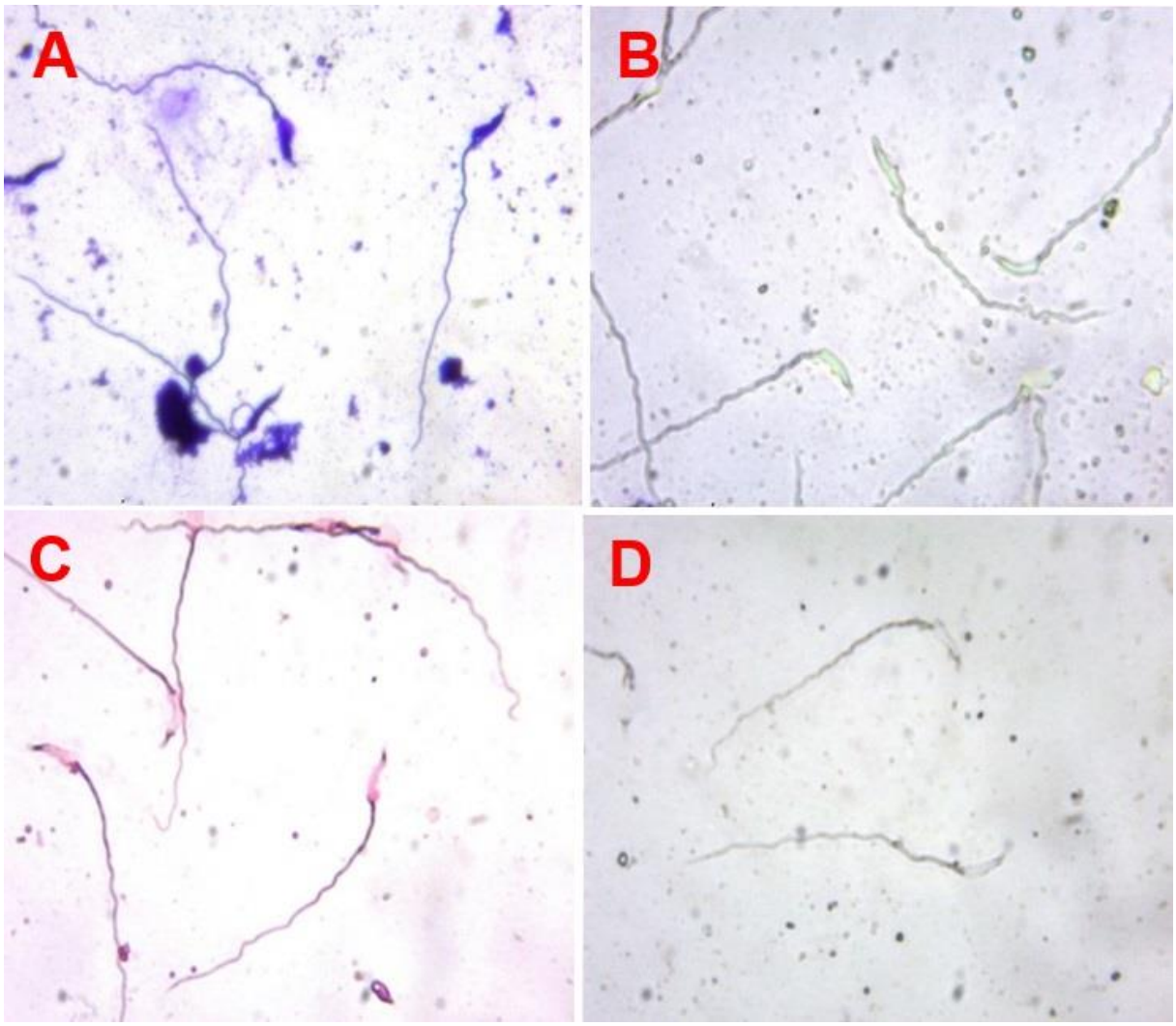

Plate 4: Photomicrograph of Lizard spermatozoa stained with aqueous extract of *Lawsonia inermis* leaf showing complete staining of the head in A, B & C with an outline of the head in D. A= Crystal violet stain, B= *Lawsonia inermis*, C= *Lawsonia inermis* counter-stained with eosin, D= *Lawsonia inermis* counter-stained with haematoxylin, x400 magnification.

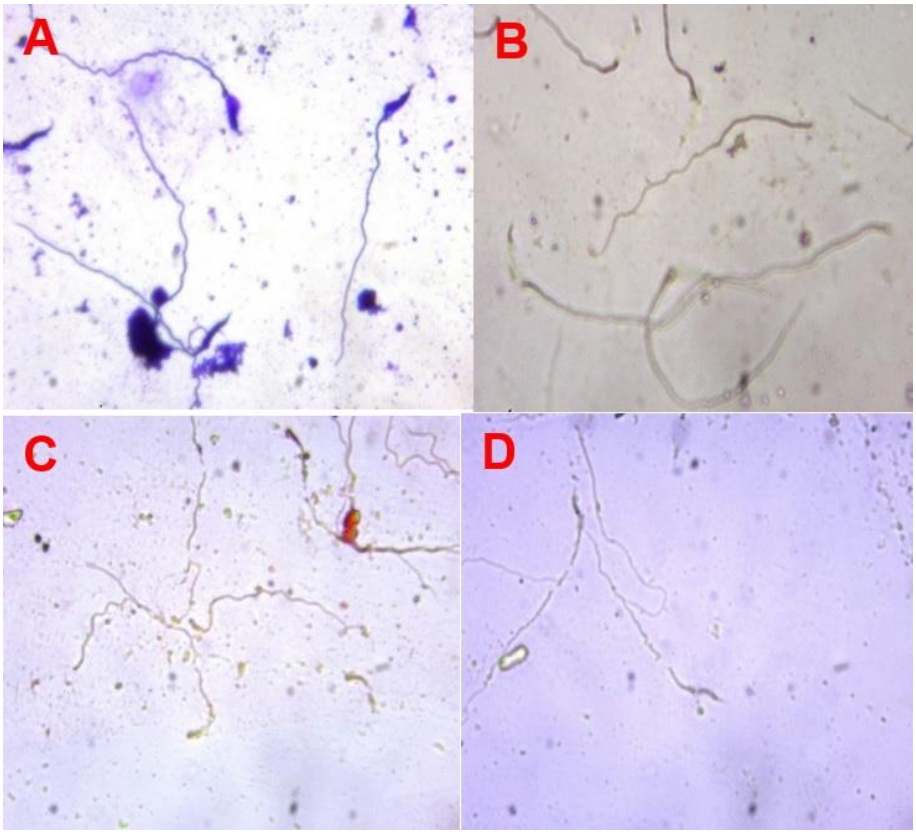

Plate 5: Photomicrograph of Lizard spermatozoa stained with aqueous extract of *Bougainvealea spectabilis* flower showing complete staining of the head in A with only tail staining in B, C, & D. A= Crystal violet stain, B= *Bougainvealea spectabilis*, C= *Bougainvealea spectabilis* counterstained with eosin, D= *Bougainvealea spectabilis* counter-stained with haematoxylin, x400 magnification

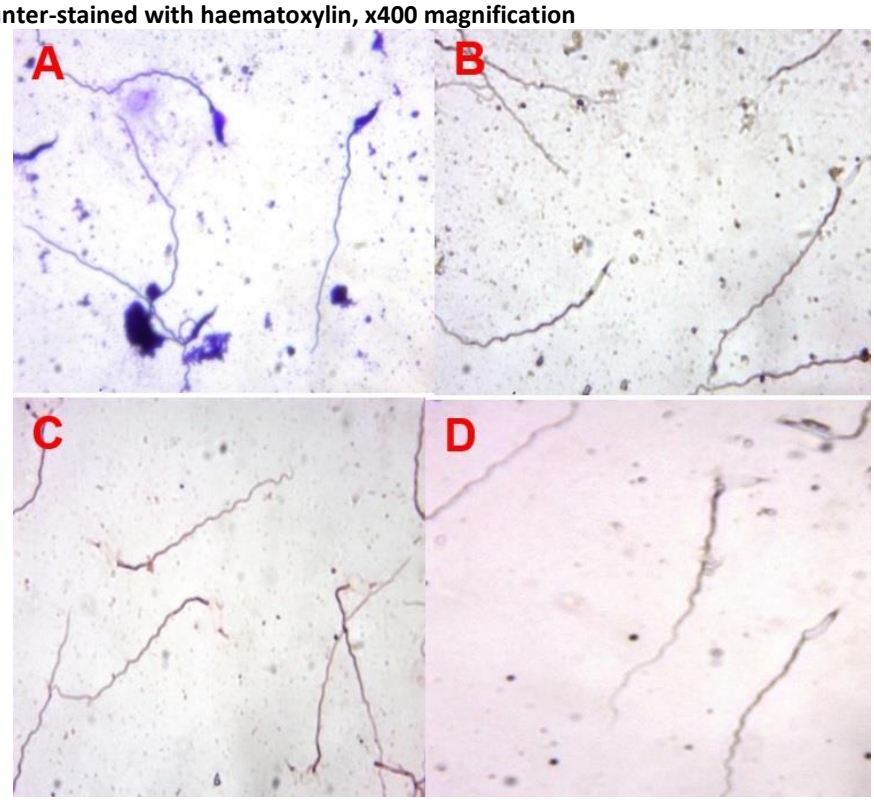

Plate 6: Photomicrograph of Lizard spermatozoa stained with *Hibiscus sabdariffa* calyx showing complete staining of the head and tail in A with an outline of the head in D and only tail staining in B & C. A= Crystal violet stain, B= *Hibiscus sabdariffa*, C= *Hibiscus sabdariffa* counter-stained with eosin, D= *Hibiscus sabdariffa* counter-stained with haematoxylin, x400 magnification

DISCUSSION

The current study revealed both head and tail staining of spermatozoa in mice spermatozoa when LIL, BSF, and HSC were counterstained with eosin. This suggests that these stains can be used in cytology and histological studies to assess the morphology of mice spermatozoa and/or human spermatozoa during sperm assessment. However, only LIL-stain spermatozoa demonstrated both the head and tail in lizards suggesting that the other stains cannot be used for sperm morphology in lizards. The tendency of all the extracts to demonstrate spermatozoa morphology in mice when counterstained with eosin suggests that any of these stains can be used in sperm morphological studies in rodents. Rodents constitute over 90 percent of all laboratory animals with mice as the most commonly used animal in biomedical research (Hickman *et al.,* 2017).

The current study showed that sperm staining with the aqueous extracts of *Lawsonia inermis* leaf, *Bougainvealea spectabilis* flower, and *Hibiscus sabdariffa* calyx showed complete staining of spermatozoa tail (cytoplasm) in mice and lizards. This is similar to a previous work by Bossey *et al.* (2012) who reported that natural stains from *Hibiscus sabdariffa, Lawsonia inermis,* and *Bougainvillea* stained the cytoplasmic components of the tissues similar to haematoxylin and eosin staining. It is also in line with the work of Deepali *et al.* (2014), who observed better cytoplasmic staining when animal tissues were stained with watery extract of *Lawsonia inermis* leaves, *Hibiscus rosa-sinensis flower*, *R. indica* flower, and *Bougainvillea glabra.* Okolie *et al.* (2021) reported that the staining potentials of indigenous *Hibiscus sabdariffa* calyx, *Lawsonia inermis* leaves, and *Vitex doniana* stem barks on liver and kidney tissues showed no nuclear staining. The present work was contrary to the study by Ebrahimi and Parham (2022) who reported that henna dye could not stain any part of the sperm cells. However, several studies demonstrated that henna is an effective dye for staining different animal tissues with no morphological changes (Alawa *et al.,* 2015). *Bougainville spectabilis, Hibiscus sabdariffa, and Lawsonia inermis* showed a complete staining of the head and tail when counterstained with haematoxylin and eosin. This is similar to the work done by Kondracki *et al.* (2017) who reported that haematoxylin and eosin are excellent stains for human spermatozoa morphology as they could stain the head, acrosome, and tail with a clear distinction between all the parts.

CONCLUSION

The aqueous extracts of *Lawsonia inermis* leaf, *Bougainvillea spectabilis* flower, and *Hibiscus sabdariffa* calyx demonstrated both head and tail morphology of the spermatozoa in mice when counterstained with eosin. However, *Bougainvillea spectabilis* flower and *Hibiscus sabdariffa* calyx stains did not reveal the head morphology in lizards even with counterstaining. This suggests that extracts could be used as a morphological stain in sperm assessment instead of haematoxylin.

Conflict of Interest

The authors declared no conflicts of interest.

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