https://dx.doi.org/10.4314/dujopas.v10i4b.22
ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 10 No. 4b December 2024*

Demonstration of Oestrous Cycle Phases in Rats Using Aqueous Extract of *Bougainvillea Spectabilis* flower, *Hibiscus Sabdariffa* calyx and *Lawsonia Intermis* leaf.

Salomi Sunyarari Simon, Nathan Isaac Dibal, Sani Hyeldima Garba, Joseph Vandi Zirahei, Sunday Joseph Manye, Zainab Muhammad Goni.

Department of Human Anatomy,
Faculty of Basic Medical Sciences,
University of Maiduguri,
P.M. B 1069,
Nigeria.

Email: salomi.simon85@gmail.com

Abstract
Methylene Blue is a synthetic dye that is positively charged and binds to a negatively charged components of the cells such as DNA and RNA present in the cytoplasm, it is a cationic stain, that is used to stain both plant and animal cells. Methylene blue when not properly managed it could pose serious threat to human's health and to the environment. The dye is known to be cost effective. Since the stain is a synthetic dye, it can be harmful and not readily available, and development of natural histological stain is justified if the natural stain is cheaper, harmless and readily available. Therefore, the aim of the study was to evaluate the oestrous cycle phases in rats using Bougainvillea spectabilis flower, Hibiscus sabdariffa calyx and lawsonia intermis leaf stains as an alternative to methylene blue. At proestrus phase of oestrous cycle stained with methylene blue, Bougainville spectabilis flower, Hisbiscus sabdariffa calyx and Lawsonia intermis leaf showed numerous nucleated epithelial cells and few cornified cells and the cells. At estrus phase of oestrous cycle stained with methylene blue Bougainville spectabilis, Hisbiscus sabdariffa and Lawsonia intermis stained showed enucleated cells. At Metestrus phase, all the stains showed leucocytes, irregular cells, enucleated and some nucleated cells. Our findings showed that Hibiscus sabdariffa calyx stain demonstrated proestrus and estrus phases, Bougainvillea spectabilis flower demonstrated estrus and metestrus phase while lawsonia intermis leaf only demonstrated estrus phase. Hibiscus sabdariffa calyx can be used as a local stain in researches that involve mating of rat.

Keywords: *Bougainville spectabili, Hisbiscus sabdariffa, Lawsonia intermis,* Methylene blue and Oestrous cycle,

INTRODUCTION
The most common animals used in reproductive cycle researches are rats and mice, this is because they are readily available, easy to breed and handle as well as their short oestrous cycles ((Ajayi and Akhigbe 2020). In estrous cycle there are four phases known as proestrus, estrus, metestrus, and diestrus, which lasts four days, and can be identified based on the cell types found in the vaginal smear (Marcondes *et al.,* 2001). Three types of cells found in estrous cycle which are nucleated epithelial cells, cornified cells, and leukocytes seen in the vaginal smear and are used to characterize each phase. It often takes one to two hours or longer to collect vaginal discharge and employ stained material (Mclean *et al.,* 2012; Cora *et al.,* 2015).

Author for Correspondence

When nucleated epithelial cells are present, they can be easily identified as being in proestrus. Proestrus normally lasts for 14 hours, although during that time the hormone profile changes quickly (Sato *et al*., 2016). The massive, keratinized squamous cells that characterize proestrus are replaced by non-nucleated cells during estrus, which lasts for 24–48 hours. Throughout oestrus, progesterone and estradiol secretion is steady at a low level (Kinder *et al.,*1996; Deka *et al*., 2007). Diestrus is the phase with the longest duration and most classification inconsistencies. Leucocytes are abundant in smears during the diestrus period, but the quantity, nature, and presence of other cell types and mucus fluctuates. The majority of employees divide diestrus into two periods, which are also known as metestrus and diestrus, diestrus I and II, or early and late diestrus, as was previously noted (Marcondes *et al.,* 2001; Pantier *et al.,* 2019).

Bougainvillea spectabilis wild (BS) referred to as great bougainvillea is a woody vine species that belong to the Nyctaginaceae family and was discovered first by a French navigator in Brazil named Louis Antoine bougainvillea in 1786 (Kobayashi, *et al.,* 2007). Traditionally, Bougainvillea spectabilis has been used to treat various ailments such as respiratory infections, gastrointestinal disorders, and skin conditions (Al-Snafi, 2015). The pigment betacyanins of Bougainvillea flowers' bract exhibit not only a positive health benefits, but also act as a good coloring agent (Nur *et al.,* 2016).

Hibiscus sabdariffa (HS) is a plant that belong to the vascular flowering plants, known as Roselle or Red Sorrel in English, the plant is grown in many countries around the world, including Sudan (Sahel *et al.,* 2023) In Sudan, it is known as Karkade and in Nigeria it is popularly called zobo (Mohammed *et al.,* 2012). The plant has a variety of well-known industrial, medical, and nutritional applications (Odigie *et al.,* 2003). It has been used as a natural stain for specimens of skin (Raheem *et al.,* 2015), kidney, liver (Okolie *et al.,* 2021), testis (Egbujo *et al.,* 2008) and fungi. Results revealed that the extract can be used as cytoplasmic stain in place of eosin in routine H and E staining.

Lawsonia intermis (LI) also known as henna is a perennial plant or a shrub that belongs to the *Lythraceae* family (Barde *et al.,* 2021). It is seen in sub-tropical and tropical areas of the Middle East, Africa, Southern Asia, Northern Australia, and other semi-arid areas, the plant is commonly called Laali in Yoruba, Lalle in Hausa, Nchanwu in Igbo and Nalle in Kanuri (Odigie *et al.,*2022). The staining ability of henna is attributed to an aromatic compound napthaquinone named as lawsone, the lawsone is also known as henno-tannic acid which are the source of the reddish-brown dye used as a cosmetic agent found in the dried leaves in large quantity (Barde *et al.*, 2021). The leaves of henna are used as a natural dye in dyeing of hands, nails, fingers and even hair (beautification). Traditionally in India it is used as fertility agent (Fareha, 2016).

MATERIALS AND METHODS

Collection and Authentication of Plant Materials

The flowers of *Baugainvillea Spectabilis* Wild were collected around the school premises of University of Maiduguri, Borno state, Nigeria while *Hibiscus Sabdariffa* calyxes and *Lawsonia intermis* leaf were purchased in the Borno modern market. They were identified and authenticated by a taxonomist at the Department of Pharmacognosy, Faculty of Pharmacy University of Maiduguri (UMM/FPH/LYR/001, UMM/FPH/MAV/002, and UMM/FPH/NYG/001) respectively.

Aqueous Extraction of the Plants

The flowers of *Baugainvillea Spectabilis* Wild, *Hibiscus Sabdariffa* calyxes and *Lawsonia intermis* leaf were air-dried and pulverized. The particle (486g of each) was dissolved in 10 litres of distilled water at (5-8^{o} C) for two days. The mixture was filtered and the filtrate evaporated to dryness in an oven at 45^{o} C.

Ethical Consideration

The research was approved by the department of Human Anatomy ethical committee, university of Maiduguri (UM/HA/UGP23.24-005) and carried out according to the national institute of Health Guide for the Care and Use of Laboratory Animals. The animals were anaesthetized with ketamine injection before dissection to minimize suffering.

Experimental Animal

Three (3) female Wistar rats (7-9 weeks old) were obtained from Department of Biochemistry animal house, University of Maiduguri, Nigeria. They were housed in plastic cages and had free access to a standard pelleted feed and water.

Sample Collection and Staining

Vagina fluid was collected from each rat by flushing the vagina with normal saline using micropipette. The samples were smeared on microscopic slides, allowed to dry and fixed in alcohol. The smear was stained with methylene blue, aqueous extract of *Bougainville spectabilis* flower, *Hisbiscus sabdariffa* calyx *and Lawsonia intermis* leaf for 5 minutes each. The slides were grouped into three and stained with *Bougainvillea spectabilis, Hibiscus sabdariffa, lawsonia intermis,* and methylene blue respectively for 5 minutes. The slides were rinsed with water, allowed to dry and observed with a light microscope.

RESULTS

Histological Observation

Micrograph of the proestrus phase stained with methylene blue and HS calyx showed nucleated and cornified cells (Figure 1A & C). However, the proestrus phase stained with BS flower and LI leaf showed irregular cells that are unidentifiable (Figure B &D). The micrograph of estrus phase showed large irregular anucleate cells in the slides stained with methylene blue, and aqueous extract of BS flower, HS calyx and LI leaf (Figure 2A-D). The micrography of metestrus phase stained with methylene blue, BS and LI showed numerous leucocytes and anucleate cells (Figure 3A, B &D) while those stained with HS calyx only showed anucleate cells (Figure 3C)

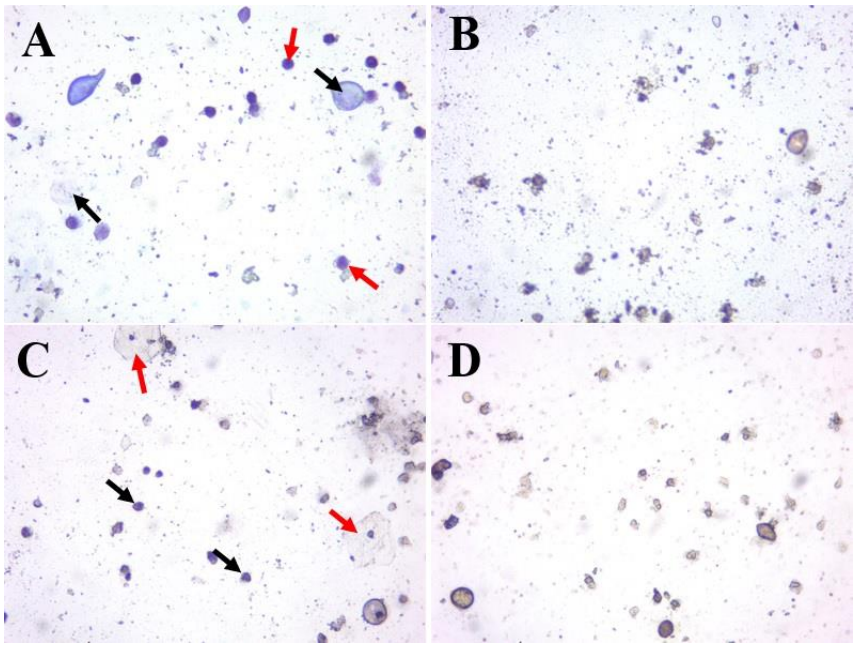

Figure 1: Micrograph of the oestrous cycle at proestrus phase showing nucleated cells (red arrow) and cornified cells (black arrow) in A & C. The cells were not identifiable in B & D. A=Methylene blue B=*Bougainville spectabilis* flower, *C= Hisbiscus sabdariffa* calyx, *D= Lawsonia intermis* leaf. x200 magnification.

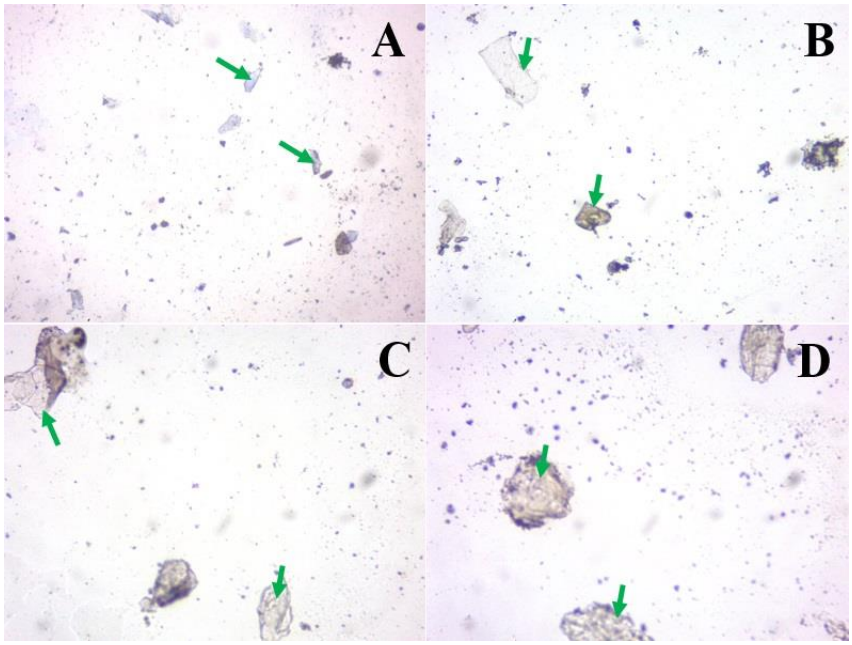

Figure 2: Micrograph of the oestrous cycle at estrus showing anucleate cells (green arrow) in A-D. A=Methylene blue B=*Bougainville spectabilis* flower, *C= Hisbiscus sabdariffa* calyx, *D= Lawsonia intermis* leaf. x200 magnification.

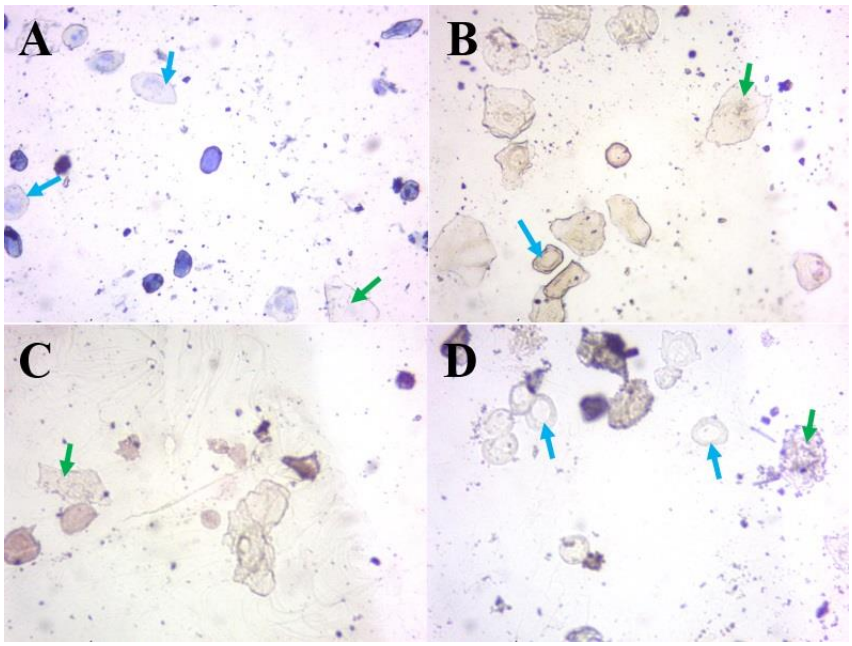

Figure 3: Micrograph of the oestrous cycle at metestrus showing leucocytes (blue arrow) in A, B & D and irregular anucleate cells (green arrow) in A-D. A=Methylene blue B=*Bougainville spectabilis* flower, *C= Hisbiscus sabdariffa* calyx, *D= Lawsonia intermis* leaf. x200 magnification.

DISCUSSION

Oestrous cycle is a stage that identify the presence, absence, or proportion of three types of cells, nucleated epithelial cells, cornified cells and leucocytes, the cycle is divided into four stages of proestrus, estrus, metestrus and diestrus (Michelle *et al.*, 2015). The changes observed in the vaginal cytology are the indication of the endocrine events (Ekambaram *et al.*, 2017), the proportion of cells type define the stage of estrous cycle in rats. In the proestrus stage, when mature epithelial cells are shed and predominate in the vaginal smear, during this stage overall blue cast is due to methylene blue, which stains the cytoplasm's of mature squamous cells blue. methylene blue is a polychromatic stain which impart color to both nucleus and cytoplasmic details. The methylene blue stain which aids in preserving the smears, and cells are easily described due to their consistent staining pattern (Ekambaram *et al.*, 2017). In estrus, the large cornified cells appear blue due to keratin, which is synthesized during cell differentiation (Srinivasan *et al.*, 2017). In metestrus clumping of leukocytes around the epithelial cells are likely due to the action of chemokines, specialized molecules that bind leukocytes in tissues (Townson and Liptak 2003). Chemokines are expressed as a result of luteal regression and are associated with accumulation of leukocytes (Townson and Liptak 2003). In proestrus phase, Methylene blue and aqueous extract of *Hibiscus sabdariffa calyx* showed detailed nucleus and cytoplasm staining but not clearly differentiated in aqueous extract of *Bougainvillea spectabilis* flowers and *Lawsonia intermis* leaf. Pre-acidophilic cells were maximal during proestrus, making the identification of this phase very easy in stained smears. Other researcher reported that leucocytes were found in all stages of oestrous cycle (Paccola *et al.*, 2013). However, leucocytes were not detected during the proestrus and estrus phase in our study. These results are similar to those obtained by various authors (Ajayi and Akhigbe 2020; Bakare *et al.*, 2022) who noticed leucocytes only during the metestrus and diestrus phase. In estrus phase, the smears stained with methylene blue, aqueous extract of *Bougainvillea spectabilis* flower, *Hibiscus sabdariffa* calyx and *Lawsonia intermis* leaf showed anucleate cells in all. It is in line with the study conducted by Odigie *et al.*, (2022) that *Lawsonia intermis* is not effective in nuclear staining of some tissues (kidney, lungs, intestine and tonsil). The metestrus phase showed leucocytes, irregular anucleate cells, and few nucleated cells when stained with

methylene blue, aqueous extract of *Bougainvillea spectabilis* flower, *Hibiscus sabdariffa* calyx and *Lawsonia intermis* leaf. In the present study, we hypothesized that the aqueous extracts of *Hibiscus sabdariffa* calyx and *Lawsonia intermis* leaf can be called cytoplasmic stains since they all present a clear cytoplasmic stain with no nuclei staining. The result of this study is in line with the work of Deepali *et al.* (2014), who observed better cytoplasmic staining when animal tissues were stained with the aqueous extract of *Hibiscus sabdariffa* and *Lawsonia intermis, Bougainvillea spectabilis.* However, it is contrary to the work done by Sahel *et al.* (2023) who also reported that *Hibiscus sabdariffa* has a good nuclear staining for buccal and cervical pap smear. Paccola *et al.* (2013) reported that at metestrus phase, the presence of leucocytes has been associated with high progesterone levels, which induce influx of neutrophils into the vagina. Johnson (2007) also reported that in metestrus phase the vagina smears exhibited clumped of anucleate cells and is similar to those observed during the estrus phase. Although, few leucocytes are found in smear during metestrus enabling differentiation between the estrus and metestrus phases.

CONCLUSION

Our findings showed that aqueous extract of *Hibiscus sabdariffa* calyx may be use as a local stain in the demonstration of proestrus and estrus phase in rodents. Hence, it can be employed in research that involve mating of rat since proestrus and estrus are important in estimating mating period.

Acknowledgements

Authors are grateful to members of Department of Histology Laboratory, University of Maiduguri for their assistant in numerous ways.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCE

Ajayi, F.A. and Akhigbe, R.E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertility Research and Practice,* **6**(5):1-5.

Al-Snafi, A. E. (2015). Therapeutic properties of medicinal plants:Na review of their detoxification capacity and protective effects. *Journal of pharmaceutical science and technology,* **5**(1):257-270.

Bakare, A. A., Idaguko, C. A., Sogbesan, Z.A. and Adeoye, F. (2022). The effects of variations in length of suction pipette inserted during vaginal smear cytology procedure on estrous cycle in Sprague-Dawley rats. *Journal of Biological Research and Biotechnology*, **20**(2):1600-1605.

Barde, I. J., Kamdip, S.N., Gushe, D. D. and Dashe, Y. G. (2021). Assessing the use of *lawsonia inermis* and *Hibiscus sabdariffa* Aqueous Extracts as a Possible Substitute for Eosin Stain in Paraffin-embedded Tissues. *Journal of Medical Science and Academic Research,* **2**(2):5-12.

Cora, M.C., Kooistra, L. and Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicology Pathology*, **43**(6):776– 793.

Deepali, K., Lalita, S. and Deepika, M. (2014). Application of aqueous plant extracts as biological stains. International journal of science and engineering research, 5(2):1586-1589 Gronroos, M. and Kaupilla, O. (1959). Hormonal-cyclic changes in rats under normal conditions and under stress as revealed by vagina smears after Shorr staining. *Acta Endocrinology* (Copenh), **32**(2):261-271.

Deka, K.C., Borgohain, Nath, K.C., Goswami, J. and Biswas, R.K. (2007). A comparative appraisal of serum progesterone level during oestrous cycle in normal and repeat breeder crossbred cows with ovulatory problem. *The Indian Journal of Animal Reproduction*, 28(2):83-85.

Egbujo, E.C., Adisa, O.J. and Yahaya, A.B. (2008). A Study of the Staining Effect of Roselle (*Hibiscus sabdariffa*) on the Histologic Section of the Testis. *International Journal of Morphology*, 26(4):927–30.

Ekambaram, G., Kumar, S., Kumr, S. and Joseph, L.D. (2017). Comparative study on the estimation of estrous cycle in mice by visual and vaginal lavage method. *Journal of Clinical and Diagnostic Research,* **1**(1):05-07.

Fareha, M. (2016). Effect of Aqueous Extract of *lawsonia Inermis* Leaves on The Liver Following Acute Ethanol-Induced Hepatic Damage in Wistar Rats. Ph.D. Thesis, Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria Nigeria.

Gronroos, M. and Kaupilla, O. (1959). Hormonal-cyclic changes in rats under normal conditions and under stress as revealed by vagina smears after Shorr staining. *Acta Endocrinology* (Copenh), **32**(2):261-271.

Johnson, M.H. (2007). Essential reproduction. 6th edition New York NY; Wiley-Blackwell 316p.

Kinder, E.J., Kojima, Bergfeld, F.N., Wehrman, M.E. and Fike, K.E. (1996). Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicle in Cattle. *Journal of Animal Science,* 74:1424-1440.

Kobayashi, K.D., McConnell, J. and Griffs, J. (2007). *Bougainvillea.* College of tropical agriculture and human resources. *Ornamentals of Flowers,*38(8):1-3.

Marcondes, F. K., Bianchi, F. J. And Tanno, A. P. (2001). Determination of the estrous cycle phases of rats: some helpful considerations: *Brazilian Journal of Biology-Revista Brasleira de Biologia,* 62(4A):609-614.

Mclean, A.C., Valenzuela, N., Fai, S. and Bennett, S.A.L. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *Journa of Viualization Experiments,*15(67):4389.

Michelle, C.C., Linda, K. and Greg, T. (2015). Vaginal cytology of the laboratory rats and mouse review and criteria for staging of estrous cycle using stained vaginal smear. *Toxicologic Pathology,* 43(6):776-793.

Mohamed, B.B., Sulaiman, A.A. and Roselle, A. A. (2012). (Hibiscus sabdariffa L) in Sudan, Cultivation and Their Uses. *Bulletin of Environmental Pharmacology and Life Science,* **1(**6):48–54.

Nur, A. S., Mimi, S.A.M. and Zularisam, A.W. (2016). Effect of extraction condition of natural dye pigment from bourgainvillea flowers bract. *Australian Journal of Basic and Applied Sciences,* **10**(7):172-175.

Odigie, E. B., Ehimigbai, R. O. A., Aliu, H. S. (2022). *Lawsonia inermis Linn* (Henna) Lyophilized Extracts as Alternate. Stains May Deter Staining Efficacy in Histology Sections. Dutse *Journal of Pure Applied Science,* **8**(3):1–7.

Odigie, E.B., Ehimigbai, R.O.A. and Aliu, H.S. (2022). *Lawsonia inermis* (Henna) Lyophilized extracts as alternate stains may deter staining efficacy in histology sections. *Journal of Pure and Applied Science*, **8**(3a):1-7.

Odigie, I.P., Ettarh, R.R., Adigun, S.A. (2003). Chronic administration of aqueous extract of Hibiscus sabdariffa attenuates hypertension and reverses cardiac hypertrophy in 2K-1C hypertensive rats. *Journal of Ethnopharmacology*, **86**(2–3):181–185.

Okolie, N., Aa, N.and Ib, E. (2021). Staining Potentials of Indigenous *Hibiscus sabdariffa* Calyx, *Lawsonia inermis* Leaves and Vitex Doniana Stem Barks on Liver and Kidney Tissues. *Journal Cytological Histology,* **12**(3):1–9.

Paccola, C.C., Resende, C.G., Stumpp, Miraglia, S.M. and Cipriano I. (2013). The rat's estrous analysis cycle revisited a quantitative and qualitative analysis. *Animal reproduction*, **10**(4) 677-683.

Pantier, L., Li, J. and Christian, C. (2019). Estrous cycle monitoring in mice with rapid data visualization and analysis. *Bio-Protocol,* **9**(17): e3354.

Raheem, E.M.A., Ibnouf abd, A.O., Shingeray, O.H. and Farah, H.J. (2015). Using of Hibiscus Sabdariffa extract as a natural histological stain of the Skin. *American Journal of Research Communication,* ***3***(5):3-5.

Sahel, E.G.O., Nusaiba, M, A.A., and Eiman, M. E. (2023). *Hibiscus sabdariffa* as nuclear stain for cytological buccal and cervical smear. *International Journal of Current Science Research,* **06**(1): 561-566.

Sato, J., Mssahiro,N. and Minoru, T. (2016). Comparative histopathology of the estrous or menstrual cycle in laboratory animals. *Journal of Toxicology and Pathology,* 29:155-162

Srinivasan, M.R., A Sabarinathan, A., Geetha, A., Shalini, K and Sowmiya, M. (2017) Comparative Study on Staining Techniques for Vaginal Exfoliative Cytology of Rat. *Journal of Pharmacology and Clinical Research*, **3**(3):555615

Townson, D.H. and Liptak, A.R. (2003). Chemokines in the corpus luteum: implications of leukocyte chemotaxis. *Reproductive Biology Endocrinolog,*1: 94.