

Contraceptive Effect of *Citrus Aurantifolia* Juice Vaginal Douche on Reproductive Histomorphology of Adult Female Wistar Rats

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Abstract

This study was carried out to investigate the contraceptive effect of *Citrus aurantifolia* vaginal douche on histomorphology of adult female albino Wistar rats. 25 female Wistar rats were divided into 5 groups containing 5 female per cage. 5 male rats were kept for mating. Group 2 and 3 were douched with 100% and 50 % lime concentration *Citrus aurantifolia* respectively for two weeks. Group 4 and 5 were also douched with 100 % and 50% *Citrus aurantifolia* concentration respectively for two weeks and thereafter mated. Group 1 was the control. Groups 1,2 and 3 were sacrificed, and the vagina, uterus and ovary harvested, processed to paraffin sections, cut at 5micron in thickness, stained, observed under light microscope while Groups 4 and 5 were sacrificed before the last day of gestation to determine its contraceptive effect. The results of Groups 2 and 3 showed significant distortions and inflammation of the cells associated of the ovaries, uterus and vagina. Groups 4 and 5 showed atrophy of foetal materials and fibrosis of myometrium. In conclusion, *Citrus aurantifolia* vaginal douche has deleterious effects on the female reproductive organs and foetal development.

Keywords: *Citrus aurantifolia*, Histomorphology, Vagina, Ovaries, Cervix, Uterus and wistar rat

Introduction

With recent increase in population rate due to increased birth rate all over the globe, a variety of measures have been taken to reduce birth rate due to increase poverty level, especially in developing continents such as Africa. Among these is vaginal douche believed and used by some females as a contraceptive measures to prevent diseases, unwanted pregnancy and increase good health.

A contraceptive is a substance that prevents conception/pregnancy. There are several herbal mixtures with contraceptive claims, though there are no available data on the efficacy of these plants in human's biological systems (Riddle, 1992). The annual number of conception prevented in Africa between 2001 and 2010 rose from 6.7 million to 16 million, with 9.8 million occurring in West Africa and mostly among females between ages 20 to 25 (WHO, 2011).

Vaginal douching refers to the process of washing, cleaning or rinsing the vagina with a flow of water or medicated solution with the aim of avoiding bacterial infecting the vagina, treatment of pelvic inflammatory diseases or as a contraceptive tool as in case of abortion.(Rothman *et al.*, 2003). Vaginal douching before or after sexual intercourse is a wide spread practice among women of diverse culture

worldwide, and commercial antiseptics, soaps with water and salty water are commonly use agents. Substances like lime juice, lemon juice, vinegar or acidic soft drinks in the belief that it may prevent pregnancy and /or sexually transmitted diseases; and increase sexual pleasure (Michaud, 1999). Noxynol-9(N-9), krest bitter lemon (soft drink) and lime juice have been demonstrated to have antimicrobial and spermicidal properties and have been considered as biological agents for the prevention of pregnancy and sexual transmitted infections (Roger *et al.*, 2009).

A typical example of usage is seen in cases where statistics was taken on the use of contraceptives by women and most being female sex workers douched using lemon juice as a contraceptive (Hime,1963). Roger (2000) showed that lemon and lime juice destroys sperm cells and immunodeficiency virus (HIV) due to its high acidity. He also reported its antimicrobial action against vibrio stains stating that the application of lime juice on *in-vitro* analysis shows that the juice may have anti-proliferative effects on tumour cells.

Douching has been known to have a number of supposed but unproven benefits. It is believed that aside from cleaning the vagina of unwanted odours, it can also be used by women who wish to avoid

smearing a sexual partner's penis with menstrual blood while having intercourse during menstruation (Holmes, 2004). However, many health care professionals state that vaginal douching is dangerous as it interferes with both the vaginal normal self-cleaning and with the natural bacterial culture of the vagina. It might modify vaginal flora and increase the risk of cervical infections (Atiene *et al.*, 2009). This research is therefore aimed at studying the contraceptive effect of lime vaginal douching on adult female Wistar rats.

Citrus aurantifolia commonly known as "mkpidi idem sokoro" in Ibibio. It is citrus species belonging to the family *Rutaceae*. It is a globose fruit of about 2.5 – 5 cm in diameter (1 -2 inches) that is yellow when ripe but usually picked green commercially. It is smaller and seedier with a higher acidity and a strong aroma. The tree of which the fruit is harvest is shrubby in nature of about 5 m (16 feet) with many thorns. Its trunk rarely grows straight with many branches that originate quite far down the trunk. The leaves of this plant are oval, 2.5 – 9 cm (1 -3.5 inches) long resembling orange leaves. The flowers are 2.5cm (1 inch) in diameter, yellowish white with a light purple tinge on the margins (Wiktionary, 2011).

Ecologically, *Citrus aurantifolia* is most competitive in areas that receive about 700 to 1000 mm of mean annual precipitation. It tolerates drought better than any of the other citrus fruit species (Morton, 1987). If planted, it will grow but becomes increasingly susceptible to disease in areas that receive up to about 2000 mm of annual precipitation. Most drain soil are suitable particularly those rich in calcium (Morton, 1987).

Citrus aurantifolia is intolerant of shade and will not survive long under a closed forest canopy. Cattle-grazing encourages it somewhat by eliminating some of the competition without damaging it. It is mostly killed by fire and hard frost but will sprout and survive if the disturbance is not repeated frequently (Katzer, 2002).

The most important biological active constituents of *Citrus aurantium* fruits are piperethyamine, N-methyltyramine and hordenine. It is rich in vitamin C, flavonoids and volatile oils (Suryawanshi, 2011). Others include limonene, pinene, canol, linalool, linalyl acetate, bergaptene, terpinoline, bisabobolene (Kazer, 2002; Lin, 2007).

The principal use of *Citrus aurantifolia* is still for food, refreshing drinks, tasty desserts and for seasoning meats, vegetable, salads' sauces and casseroles (Ehler, 2002; Katzer, 2002). A tea prepared from juice, fruit rind or leaves acts as an expectorant and relieves catarrh brought by cold or flu.

The mesocarp is also used as a very good facial scrub and helps in prevention of pimples due to its cleansing action on the skin (Aibinu *et al.*, 2007). Citric acid is said to have spermicidal properties and women soak sponges in lemon or lime juice and insert them vaginally as a method of birth control. The fresh fruits and bottle juice are an excellent source of vitamin C and were once relied upon to prevent scurvy (Bruneton, 1999).

Pharmacological investigations on *Citrus aurantifolia* was found therapeutically effective in application of various diseases such as its effectiveness in easy digestion, cardiovascular ailments, as anticancer, treatment of stroke, as anti-anxiety and sedative (the essential oil of *Citrus aurantifolium* contains linalools and the fragrant substance limonene have anti-anxiety and sedative effects (Carvalhi-freitas *et al.*, 2002). Antidepressant synephrine-rich citrus aurantium extracts have anti-depressant effects (Kim *et al.*, 2001; Song *et al.*, 1996), as antiviral – whole *Citrus aurantium* peels contains citral limonene and several citrus bioflavanoids including hesperidin, narigin, neohesperidins and rutin weak evidence hints that this substance might have antiviral effect.



Figure 1: Photograph showing Lime Fruit [*Citrus aurantifolia*. (www.gjournals.org)]

Douching materials, techniques, frequency, motivation and seasons can vary considerably among women who douche. The prevalence of douching has decreased since 1988, but it is still a common practice among American and African women, especially adolescents (Foch, 2000). In the United States, there have been reports of 52-69% of adolescents douching at least once and one study documenting 36% reporting douching one or more times a week (Vermund, 2001). In addition douching is prevalent in some African countries such as Nigeria, Ghana, Cote d' Ivoire where the douching rate among women has been reported to exceed 97% (Foch, 2000). The intensity and method of douching, especially douching with pressure, have been associated with adverse outcomes.

Vaginal douching with *Citrus aurantifolia* juice has been perceived among women to enhance sexual excitement through sensations of vaginal dryness, tightness and warmth (Mairiga *et al.*, 2010). It also includes to cleanse the vagina after menses before or after sexual intercourse, to prevent or treat infections such as itching due to vaginal discharge, to prevent ameliorate and vaginal odours, and less commonly to prevent pregnancy or sexually transmitted diseases (Chacko *et al.*, 1986).

Douching has been associated with many adverse outcomes. This include pelvic inflammatory disease, tumour cells growth, bacterial vaginosis, cervical cancer, low birth weight, preterm birth, human immune deficiency virus transmission, sexually transmitted disease, ectopic pregnancy, recurrent vulvo-vaginal candidiasis and infertility (Abma *et al.*, 1997).

Therefore, the risk of ascending infection from the pressure of douching may be greatest around the time of ovulation when the cervical Os is gaping and the mucus is thin (Newton, 2001).

The estrous cycle comprises of the recurring physiological changes that are induced by reproductive hormones in most mammalian females. (Wiktionary, 2009). Estrous starts after puberty in sexually mature female Wistar rats and are only interrupted by some physiological changes such as pregnancy (Wiktionary, 2009)

The short length of the estrous cycle of female rat makes them ideal for investigation of changes going on during the reproductive cycle. The estrus cycle last for four days and is characterised as: proestrus, estrus, metestrus, and diestrus which can precisely be determined by the observation of the cell types present in the vaginal smear (Long and Evans, 1922; Freeman, 1988). The ovulation occurs from the beginning of the proestrus to the end of estrus (Young *et al.*, 1941; Schwartz, 1964; Marcondes *et al.*, 2002). Vagina composed of inner mucosa, middle muscularis and outer adventitia. Presence of lamina propria covered by stratified squamous epithelium. The uterus is duplex comprising of two uterine horns that join together and open into the vagina via two

separate cervixes. Presence of perimetrium, myometrium (compose of inner circular and outer longitudinal layers, and endometrium (composed of blood vessels, pigmented stroma cells, endometrial glands, lamina propria and surface epithelium (columnar epithelium).

The paired ovaries of the rat are grape like structures that vary in gross appearance and size. Covering its surface is a single layer of modified mesothelium which is continuous with the broad ligaments (mesovarium) that support the ovary. The mesothelium can range from cuboidal, columnar or pseudo-stratified columnar in type but is mostly dependent on the cyclical changes during the oestrous cycle; presence of outer cortex, zona pellucida, tunica albuginea, ovarian bursa, ovarian rete, numerous follicles (primordial, primary, secondary, tertiary and graafian follicles).

Materials and Method

Drugs, Chemicals and Materials

Sodium chloride, formaldehyde, sodium trioxocarbonate V, sodium bicarbonate, xylene, 70% alcohol, 90% alcohol, absolute alcohol, distilled water, hutches, concentrate feed, syringe and hypodermic needles, EDTA treated bottles, latex hand glove, weighing scale, graduated vials, measuring tape, Wooden cages, feeders, weighing balance, masking tape, sample bottles, hand gloves, dissecting sets, dissecting board, staining rack, microtome, rubber pipettes, beakers, permanent markers, cotton wool, tissue paper, slides, Whatman filter paper, oven, water bath, cover slips, normal saline, distilled water and microscope. They were all procured from BDH Chemicals, England. All other chemicals were of analytical grade.

Collection, Breeding and Treatment of Experimental Animals

Twenty-five sexually matured female Wistar rats weighing between 265 – 295 g and five adult males weighing 270 – 300 g obtained from Department of Pharmacy animal house, University of Uyo were used. The rats were allowed for a period of two weeks (14 days) for acclimatization at the Faculty of Basic Medical Science animal house before commencement of research work. The animals were housed in wooden cages with sawdust as beddings. They were fed with the rat chow and tap water *ad libitum*. The rats were exposed to 12 hour light/dark cycle at a room temperature of 27 – 30°C. They were identified by different colour marking on their tails. All the rats were handled in accordance with the standard for the care and use of laboratory animals.

Procurement and extraction of *Citrus aurantifolia* juice

The *Citrus aurantifolia* fruits were bought from Itam market, Uyo, Akwa Ibom state, Nigeria and stored in

a refrigerator. The back of the lemon fruit were always peeled prior to the time of use and a small tip of cap cut off. The juices were squeezed out of the fruit gently into a clean beaker and drained using a Whatman No. 1 filter paper. The filtered juice were collected and stored in properly covered container inside the refrigerator.

Preparation of 50% *Citrus aurantifolia* Juice

50% concentrated lime juice were prepared by diluting concentrated lime juice with equal volume of distilled water.

Grouping of the Experimental Animals

The animals were group into six groups with five animals per cage. The cages of the various grouped rats were labelled:

Group 1: Control without treatment

Group 2: Administered concentrated *Citrus aurantifolia* juice

Group 3: Administered 50% concentrated *Citrus aurantifolia* juice

Group 4: Administered concentrated *Citrus aurantifolia* juice, then mated

Group 5: Administered 50% concentrated lime juice, thereafter mated

The male rats were used for mating.

Determination of the Estrous cycle

After two weeks of acclimatization, every morning between 8:00 a.m. and 10:00 a.m., vaginal secretions were collected from the female rats. It was done by using a plastic pipette filled with 10 μ l of normal saline and inserting the tip of the pipette into the vagina, but not deeply. Vaginal fluid was smear on glass slides and different glass slides for each animal were used in each cage. The vaginal smears were then view under the light microscope and the different cells seen on the slide under the microscope.

Administration of the *Citrus aurantifolia* to Animals

The 100% concentrated and 50% concentrated lime juice were administered to the differently grouped rats intra-vaginally by measuring 0.1 ml of the lime juice in an insulin syringe of which the needles were removed.

Necropsy Schedule

After two weeks of daily douching, groups 1 and 2 treated with 100% and 50% lime respectively were sacrificed alongside group 5 which served as control. This was done by introducing the animals one after the other into a dessicator to which a cotton wool soaked in chloroform was placed inside. After inducing the animals into an anaesthetic state, they were removed, place on the dissecting board and

dissected. The vagina, uterus and ovaries were harvested from each animal. The tissues were washed in normal saline before being fixed in 10% buffer formalin in labelled sterile bottles for a week.

Sample collection for Histopathological analysis

At the end of the stipulated days of administration of the *Citrus aurantifolia*, the rats were subjected to a 12 hours fast but had access to water and they were sacrificed using chloroform vapour. Caudal Epidydimis were carefully harvested out from the rats, harvested organs were carefully dissected out, trimmed of all fat and connective tissue blotted dry to remove any blood. The tissues were immediately fixed in Bouin's fluid transported to the Histopathology laboratory, After 72 hours, 2-3 mm in thickness were dissected out and post fixed in Neutral Buffered Saline and then transferred to a graded series of ethanol. On day 1, they were placed in 70% alcohol for 7 hours, then transferred to 90% alcohol and left in the latter overnight. On day 2, the tissues were passed through three changes of absolute alcohol for an hour each then cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58°C. Three changes of molten paraffin wax at one-hour intervals were made, after which the tissues were embedded in wax and blocked out. Prior to embedding, it was ensured that the mounted sections to be cut by the rotary microtome were orientated perpendicularly to the long axis of the kidney, liver and pancreas. The sections were designated "vertical sections". Serial sections of 5 μ m in thickness were obtained from a solid block of tissue, fixed on clean albuminized slides to prevent sections coming off the slides and later stained with Haematoxylin and Eosin staining techniques, after which they were passed through ascending grade of alcohol, cleared in xylene and mount in DPX mountant, allowed to dry at room temperature and observed Histopathologically under digital light microscope.

Photomicrography

Records of the Histological and histochemical results were obtained by photomicrography using digital photomicrographic microscope at the Gross Anatomy Research Laboratory, Department of Human Anatomy, College of Health Sciences, University of Uyo, Uyo, Akwa- Ibom, Nigeria as illustrated in Plates below.

Results

Effect of the *Citrus aurantifolia* and vaginal douche on Estrous Cycle

There was an irregular pattern in all phases of the estrous cycle: an increase in the duration of diestrus and a considerably prolonged proestrus phase, thereby delaying ovulation in the rats given 100% undiluted lime juice (Group 1) and in those given 50% diluted lime juice (Group 2) when compared with the control (Group 1).

Contraceptive Effect of *Citrus aurantifolia* Vaginal Douche

100% undiluted and 50% diluted lime juice when used as a vaginal douche for two weeks before mating (Groups 4 and 5) did not have any contraceptive effect. The fetuses in Group 3 which was administered 100% lime juice before mating were observed to be under-developed or probably resorped (Figure 2a); however group 4 which was administered 50% lime juice was observed to have well developed foetuses (Figure 2b).

Effect of *Citrus aurantifolia* Vaginal Douche on Grossing/Macroscopy of the Foetus

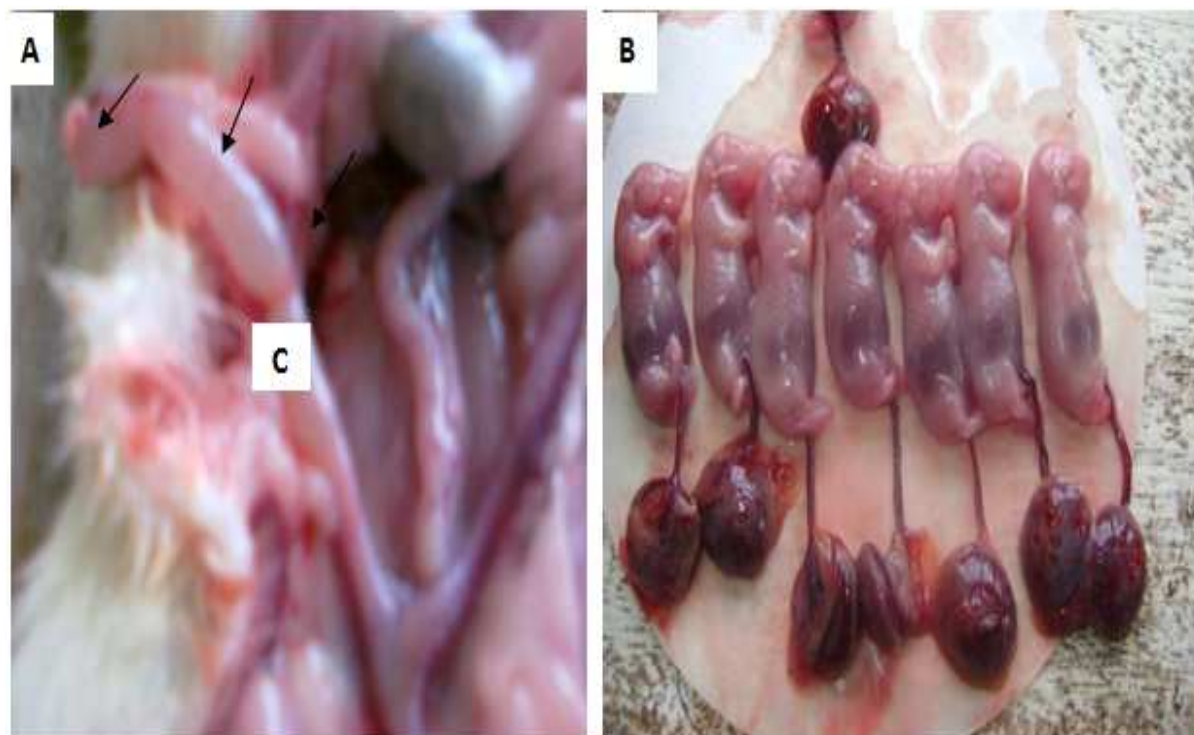


Figure 2: Photographs showing (A) under-developed or resorped foetuses in group 3 administered 100% lime juice as shown by arrows, (B) well-developed foetuses in group 4 administered 50% lime juice, (C) process of sacrifice.

Effect of *Citrus aurantifolia* Vaginal Douche on Histology of Vagina, Uterus Cervix and Ovaries

Group 2 administered with 100% *Citrus aurantifolia* juice and group 3 administered 50% lime juice showed marked area of cellular degeneration, vascular degeneration and inflammation in the vagina; with area of inflammation, vascular congestion, fibrosis and granulated eosinophilic cells in the uterus and cortical area with lining degenerated, marked cellular degeneration, follicular degeneration, atrophy and inflammation in the ovaries when compared to control.

Citrus auratifolia Vaginal Douche on Histology of Uterus stained with H&E Technique.

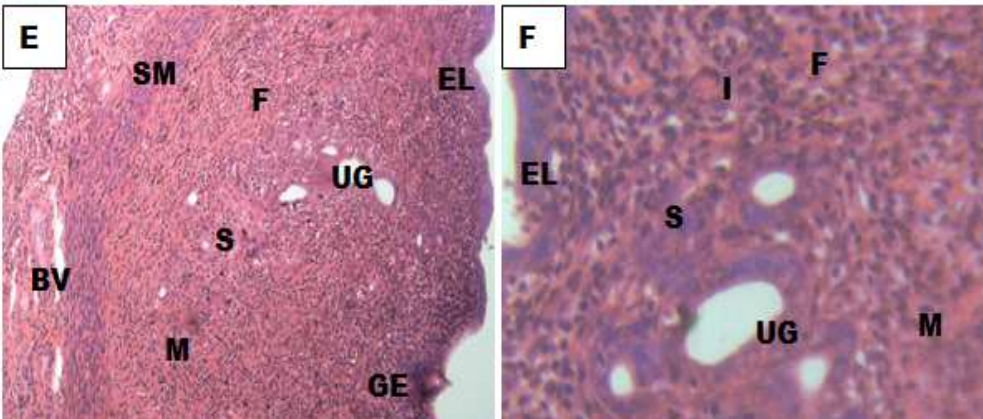
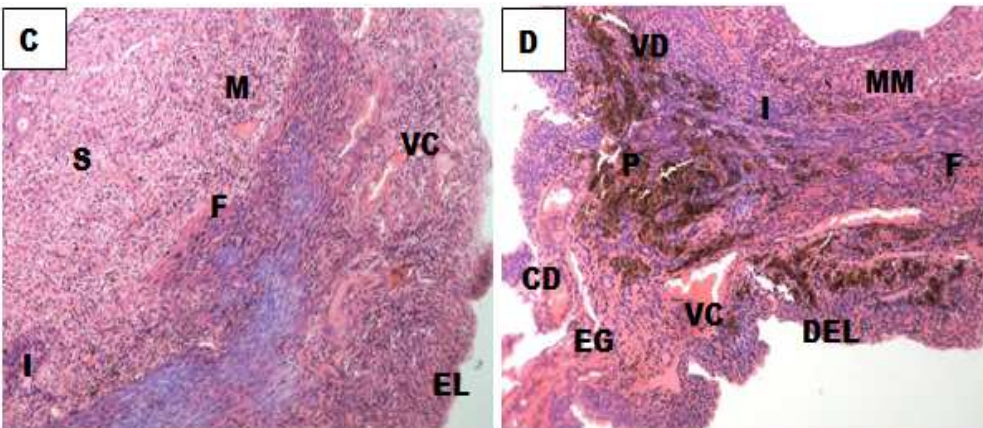
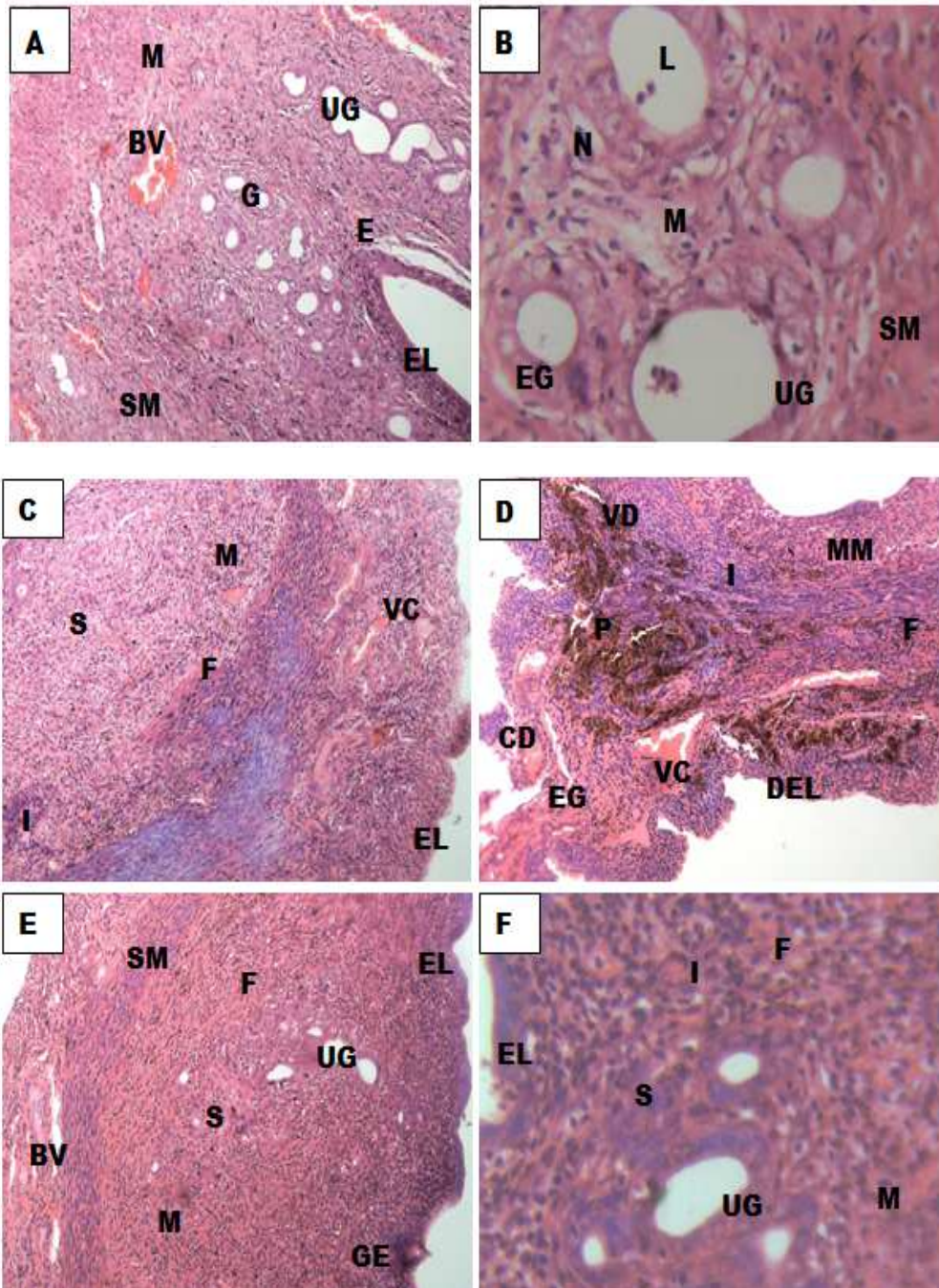


PLATE 1- A (100) & B(400) Control Uterus without administration of the *Citrus auratifolia*.

PLATE 2- C (100) & D(400) Uterus treated with 100% of *Citrus auratifolia*

PLATE 3- E (100) & F(400) Uterus treated with 50% of *Citrus auratifolia*.

Keys: BV –Blood vessels, S- Strom, EL-Epithelial lining, UG- Uterine gland, SM- Smooth muscle, M- Myometrium, E- Endometrium, F- Fibrosis, I- Inflammation, VC- Vascular congestion, CD- cellular degeneration, DEL -Epithelial lining degeneration, G-gland and L- lumen.

Citrus auratifolia Vaginal Douche on Histology of Ovaries stained with H&E Technique

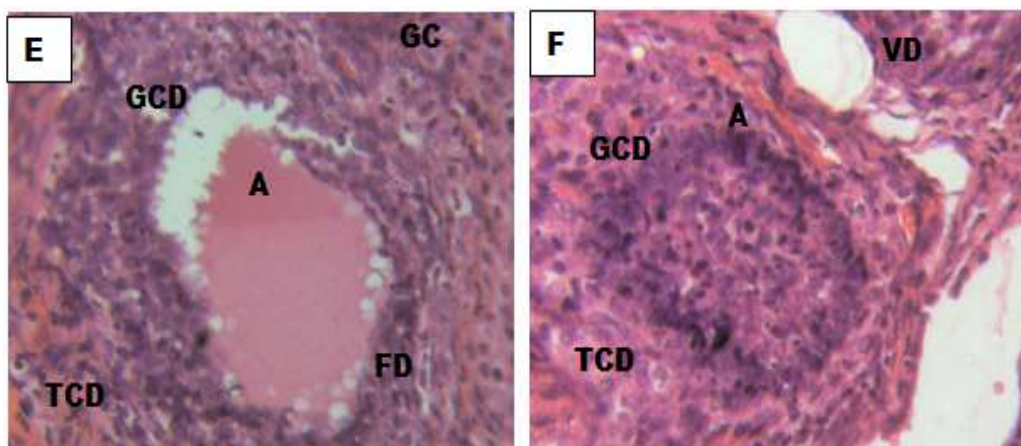
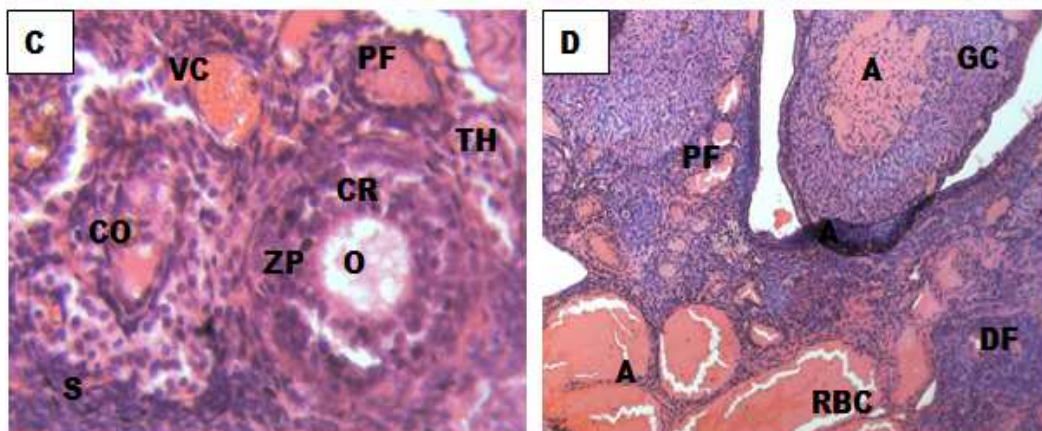
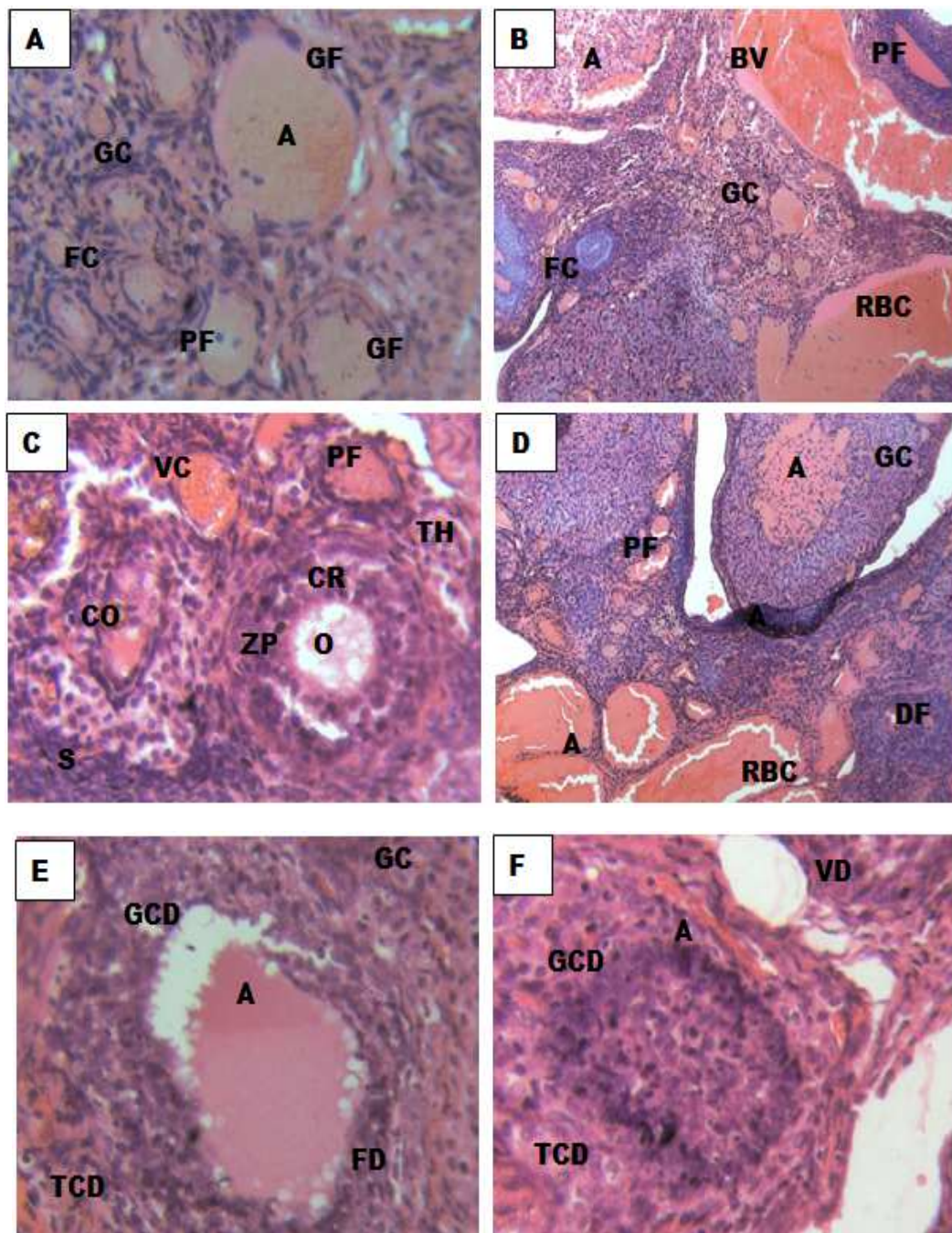


PLATE 1- A (100) & B (400) Control Ovaries without administration of the *Citrus auratifolia*.

PLATE 2- C (100) & D (400) Ovaries treated with 100% of *Citrus auratifolia*

PLATE 3- E (100) & F (400) Ovaries treated with 50% of *Citrus auratifolia*.

Keys: BV –Blood vessels, FA- follicle Antrum, GCD-Granulosa cell degeneration, TCD – Theca cell degeneration, FD- Follicular degeneration, VD—Vascular degeneration, RBC- Red Blood cells, GC- Granulosa cell , PF- Primary follicle, CO- Cumulus Oophorus, ZP- Zona pellucid, SM- Smooth muscle, TH- Theca cells, VC- Vascular congestion, CD- cellular degeneration, GF- Graffian follicle and FC- Follicular cells, CR- Corona radiate and O- Ovum

Citrus auratifolia Vaginal Douche on Histology of Cervix stained with H&E Technique.

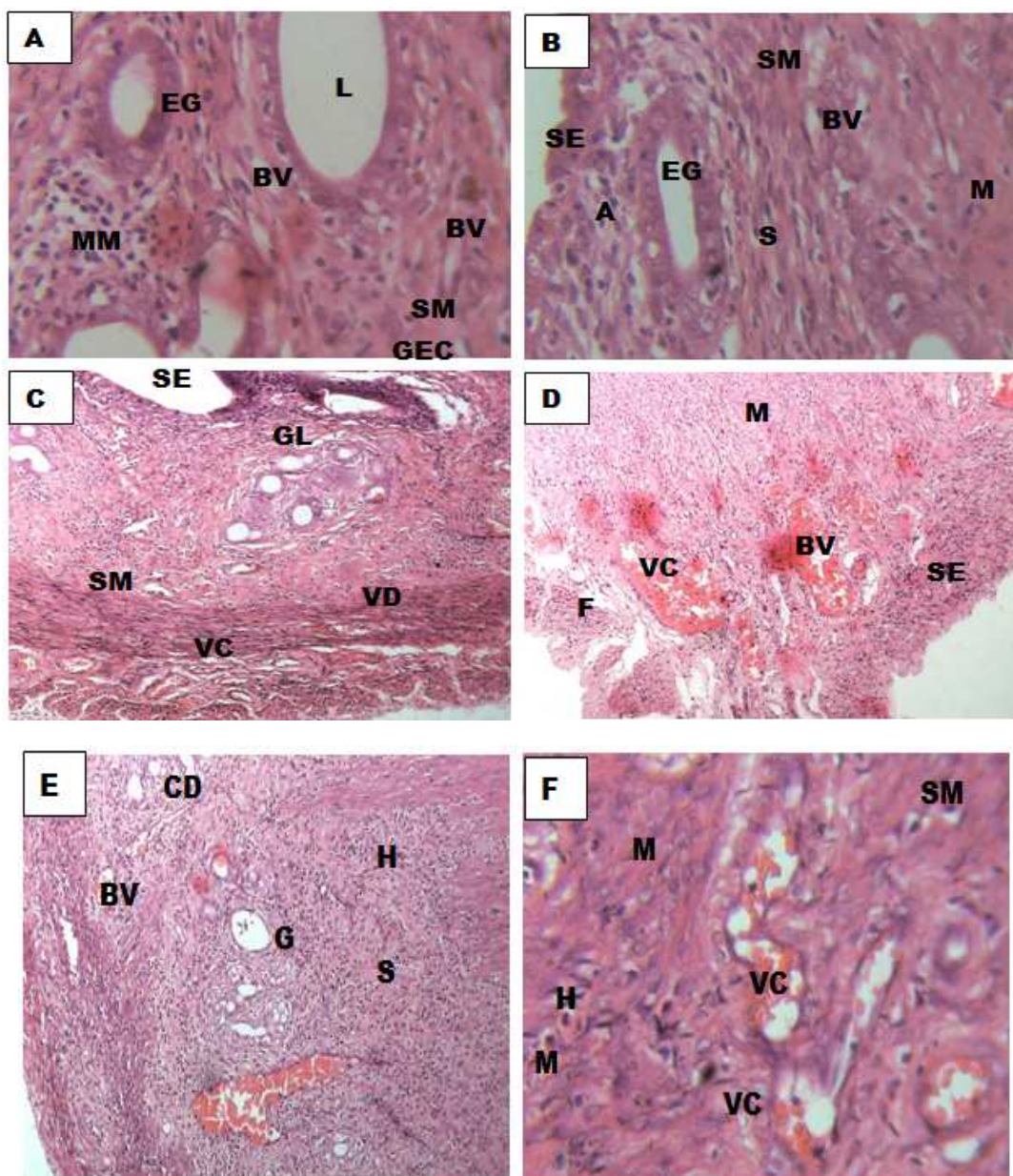


PLATE 1- A (100) & B(400) Control Cervix without administration of the *Citrus aurantifolia*.

PLATE 2- C (100) & D(400) Cervix treated with 100% of *Citrus urantifolia*

PLATE 3- E (100) & F(400) Cervix s treated with 50% of *Citrus aurantifolia*.

Keys: BV –Blood vessels, CD- Cellular degeneration, S- Stroma, El-Epithelial lining, UG- Uterine gland, SM- Smooth muscle, M- Myometrium, E- Endometrium, F- Fibrosis, I- Inflammation, VC- Vascular congestion, CD- cellular degeneration, DEL -Epithelial lining degeneration, G-gland and L- lumen. **Keys:** BV –Blood vessels, SM – Smooth muscle, BV – Blood vessel, MM – Musclaris mucosa, L – Lumen, EG – Endometrium gland, S – Stroma, GEC – Granulated Eosinophil cells, VC – Vascular congestion, SE – Squamous epithelium, VD – Vascular degeneration, H – Hyperplasia, M – Myometrium, G – Gland, F – Fibrosis.

Citrus auratifolia Vaginal Douche on Histology of Vagina stained with H&E Technique.

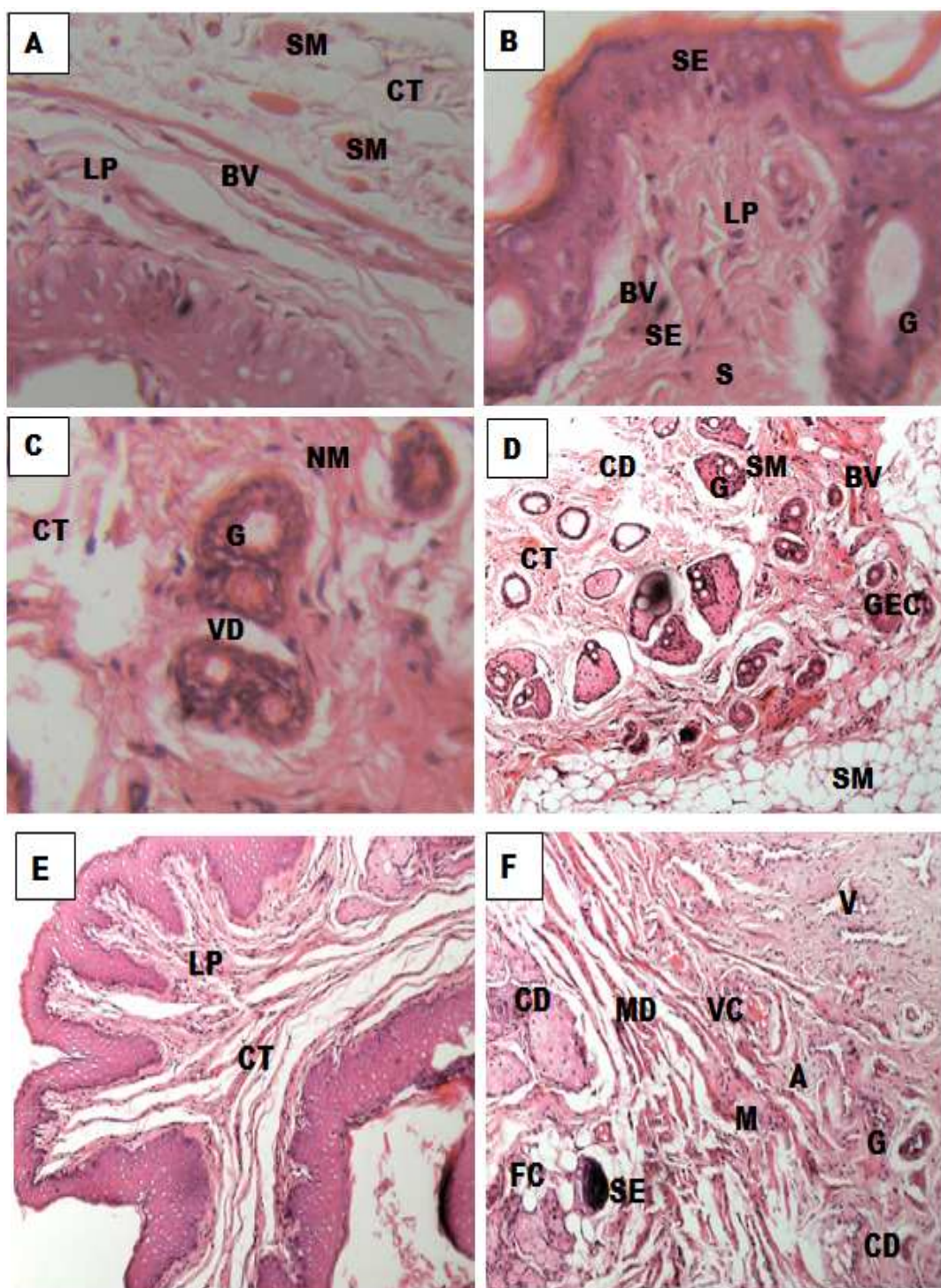


PLATE 1- A (100) & B(400) Control Vagina without administration of the *Citrus auratifolia*.

PLATE 2- C (100) & D(400) Vagina treated with 100% of *Citrus auratifolia*

PLATE 3- E (100) & F(400) Vagina treated with 50% of *Citrus auratifolia*.

Keys: BV – Blood vessels, SM – Smooth muscle, BV – Blood vessel, MM – Musclaris mucosa, L – Lumen, EG – Endomentrium gland, S – Stroma, GEC – Granulated Eosinophil cells, CT – Connective tissue, MM – Musclaris mucosa, VD – Vascular degeneration, V – Vascular degeneration, CD – Cellular degeneration, FC – Fibro muscular constriction, MD – Muscular degeneration and G – Gland.

Discussion

Lime although known for its medicinal usefulness could also exhibit adverse effect on the internal body tissues when used under prolong or continued conditions (Holmes, 2004).

Our study demonstrated that lime juice used as a vaginal douche alters the estrous cycle, by prolonging the duration of the proestrus phase and subsequently lowering the frequency at which the estrus phase occurs. Consequently the frequency of ovulation was reduced and fertility may have therefore been impaired. This suggests that lime caused an imbalance of the ovarian and extra-ovarian hormones, since it has been reported that imbalance in these hormones leads to irregularity in the ovarian functions and duration of the estrous cycle (Circosta *et al.*, 2001). Our findings are similar to that of Bakare *et al.* (2012) which concluded that lime juice taken orally may possibly compromise fertility.

The presence of substances such as citric acid could severely distort or even damage tissues or organs to which it comes in contact with, especially in cases like douching to which the acid and other chemical constituents comes in contact with the various tissue cells directly.

In this study, it was evident that administration of concentrated *Citrus aurantifolia* juice intravaginally leads to severe distortion and inflammation of the associated tissue cells. This was observed when the photomicrograph of the control was compared with that of the treated groups. Further observation was seen when all the mated rats with concentrated lime exhibited resorped foetus compared with those administered with 50% lime which had normally developed foetus. Pregnancy can be disrupted by interference with mitotic division of the foetus as is the case with cytotoxic agents, with the process of implantation which may eventually lead to pre- and post-implantation embryonic loss (Elbetieha *et al.*, 2000). Lime as a vaginal douche could have caused oxidative stress which affects early embryo development and implantation, and which in turn affects pregnancy outcome.

The vagina, uterus and ovary reacted to the administration and showed tissues that were severely inflamed and distorted after the fourteen days of administration.

5.2 Conclusion

The results of this study suggest that lime vaginal douche does not have contraceptive effect but has been found to have deleterious effect on foetal development and female reproductive histology.

Conflict Interests

The authors declared that they have no competing interests.

Authors' Contributions

All the Authors contributed equally.

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