

## **Citrus aurantifolia (Lime) Juice Negatively Influences Estrous Cycle of Wistar Rats**

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**Abstract:** *Citrus aurantifolia* (Lime) fruit is commonly consumed and known to be rich in vitamins and other important phytochemicals. The effect of Lime juice on estrous cycle was evaluated in female Wistar rats. Different groups of rats ( $n=6$  per group) with regular estrous cycles were gavaged orally with 1ml Lime juice (25, 50 or 100%) consecutively for fourteen days. A fourth (control) group was given distilled water. Vaginal lavage was analyzed daily in treated animals for ten days from the fourth day of administration to determine estrous cycle phases, but prior to juice treatment, estrous cycles of rats were established. Animal body weights in treatment and control groups were monitored. Lime juice did not alter proestrus and estrus, but induced reduction ( $p=0.0271$ ) in the duration of diestrus phase. There was equally prolonged metestrus ( $p=0.0054$ ) in animals that were exposed to Lime juice. Percentage of number of days in proestrus and metestrus were elevated after Lime juice treatment, while estrus and diestrus were decreased. This alteration in cyclicity was observed majorly in the rats that received 50 and 100% concentrations. Furthermore, Lime juice treatment enhanced body weight gain, particularly the undiluted form. The results suggest that *Citrus aurantifolia* fruit juice may alter estrous cycle and ultimately affect fertility in female rats.

**Keyword:** Estrous cycle, *Citrus aurantifolia*, diestrus, lime juice, vaginal smear

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### **I. INTRODUCTION**

Plants have played key roles as sources of drugs in traditional and orthodox medicine. And many indigenous plants have been proven to be valuable in both prevention and treatment of various ailments. With increasing realization of the health hazards and toxicities associated with the indiscriminate use of synthetic drugs and antibiotics, interest in herbal medicine is increasing globally. Affordability and perceived safety of the herbal drugs have increased their acceptability as alternates to orthodox drugs, particularly in the developing and underdeveloped countries.

*Citrus aurantifolia* (commonly called Lime in Nigeria) is a flowering plant that belongs to the Family, Rutaceae or Citrus. It is a polyembryonic and essential medicinal plant which grows in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and the West Indies. The fruit juice (which is acidic with a characteristic fragrance) is widely consumed for various health benefits. It has been shown to possess antiseptic, anthelmintic, antifungal, antibacterial, antiviral, anticancer, antioxidant and antidiarrheal activities [1-3]. The fruit of *Citrus aurantifolia* contains high amounts of ascorbic acid, minerals (calcium and potassium) and essential oils. These components, together with many other antioxidant and nutritional compounds that have been identified in the plant like flavonoids, phenols, coumarins, tannins, alkaloids, lycopene, proteins, carbohydrates, and lipids [2-4], make it an important medicinal plant. Products of Citrus fruit such as essential oil and pectin are used in the cosmetic and pharmaceutical industries [5,6].

Citrus is used locally in many countries for management of different medical conditions. In some parts of Nigeria, it is a common practice for women to use Lime juice solution as a post coital douche with the belief that it prevents pregnancy or sexually transmitted diseases (such as HIV infection), and enhances sexual pleasure [7,8]. Interestingly, it has been reported to possess abortifacient effect [9], but not much studies have been done on female reproductive effect of the plant. Its potential effect on fertility in the female is yet to be established. In the male, the fruit juice has been demonstrated in previous studies to exhibit anti-spermatogenic effect in experimental animals [10-12]. The objective of this study is to investigate the influence of *Citrus aurantifolia* fruit juice on estrous cycle in Wistar rats.

## II. MATERIALS AND METHODS

### 2.1 Plant Materials

Fresh *Citrus aurantifolia* (Lime) fruits were purchased from a green grocer (Kaduna Street, D-line, Port Harcourt, Nigeria) and identified by Dr. E. N. L. Wosu, a botanist in the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria. The fruit was washed with water, sliced and the juice was squeezed out and stored in a refrigerator (-4°C) until used for the experiment.

### 2.2 Animal Care and Handling

Female Wistar rats with an average body weight of 180±5 g were obtained from the Animal House of Department of Experimental Pharmacology and Toxicology, University of Port Harcourt, Nigeria. They were housed in plastic cages, fed daily with commercially available rodent pellets (Eastern Premier Feed Mills Ltd, Calabar, Nigeria), and given water *ad libitum*. Animal ethics and proper handling methods were strictly adhered to [13].

### 2.3 Experimental Protocol

Twenty-four rats were used in this experiment and divided into four groups containing 6 rats per group. Three of the groups were administered 1 ml of Lime juice (25, 50 or 100%) by oral gavage consecutively for 14 days (three and half cycles). The fourth group was given distilled water and used as control. Lime juice was diluted with distilled water. Estrous cycle of animals was studied from fifth day of Lime juice administration (i.e., after one 4 day cycle treatment) and continued for 10 days (i.e., two and half cycles). Estrous cycles of rats were previously established before commencement of Lime administration. Administration of Lime juice was continued for another 14 days and body weights of treated groups were compared with body weight of control group.

### 2.4 Determination of Estrous Cycle

Estrous cycle was established in animals by vaginal smear cytology as previously described [14,15]. Briefly, normal saline was drawn into the tip of a plastic pipette and inserted about 2 mm deep into the vaginal canal of the rats, and few drops were emptied. The mixture of vaginal fluid and normal saline was suctioned into the tip of the pipette. Few drops of this was used to prepare smear on a glass slide which was cover-slipped and viewed under a light microscope using 100x and 400x magnifications. Vaginal smear was analyzed daily between 9.00 am and 10.00 am consecutively for 14 days. The animals that showed three successive 4-day cycle, typically having a sequence of proestrus, estrus, metestrus, and diestrus (P-E-M-D) phases were considered having regular estrous cycle [14] and were selected for this study. Animals that show deviation from the P-E-M-D pattern, and or absence of four day cycle were considered to have irregular estrous cycles [14].

### 2.5 Statistical Analysis

Data were analyzed using student's t-test. Data analysis was performed using GraphPad Prism 5 software and values with  $p < 0.05$  were considered significant.

## III. RESULTS

### 3.1 Phases of Estrous Cycle

The different phases of estrous cycle obtained in the rats, proestrus, estrus, metestrus, and diestrus are shown in Figure 1. The proestrus phase was characterized by presence of rounded and nucleated epithelial cells (Figure 1A). The estrus phase showed presence of cornified cells (Figure 1B). The metestrus phase was characterized by presence of a combination of leucocyte, cornified and rounded epithelial cells (Figure 1C). The diestrus phase was characterized mainly by presence of leucocytes (Figure 1D).

### 3.2 Effect of *Citrus aurantifolia* (Lime) Juice on Estrous Cycle

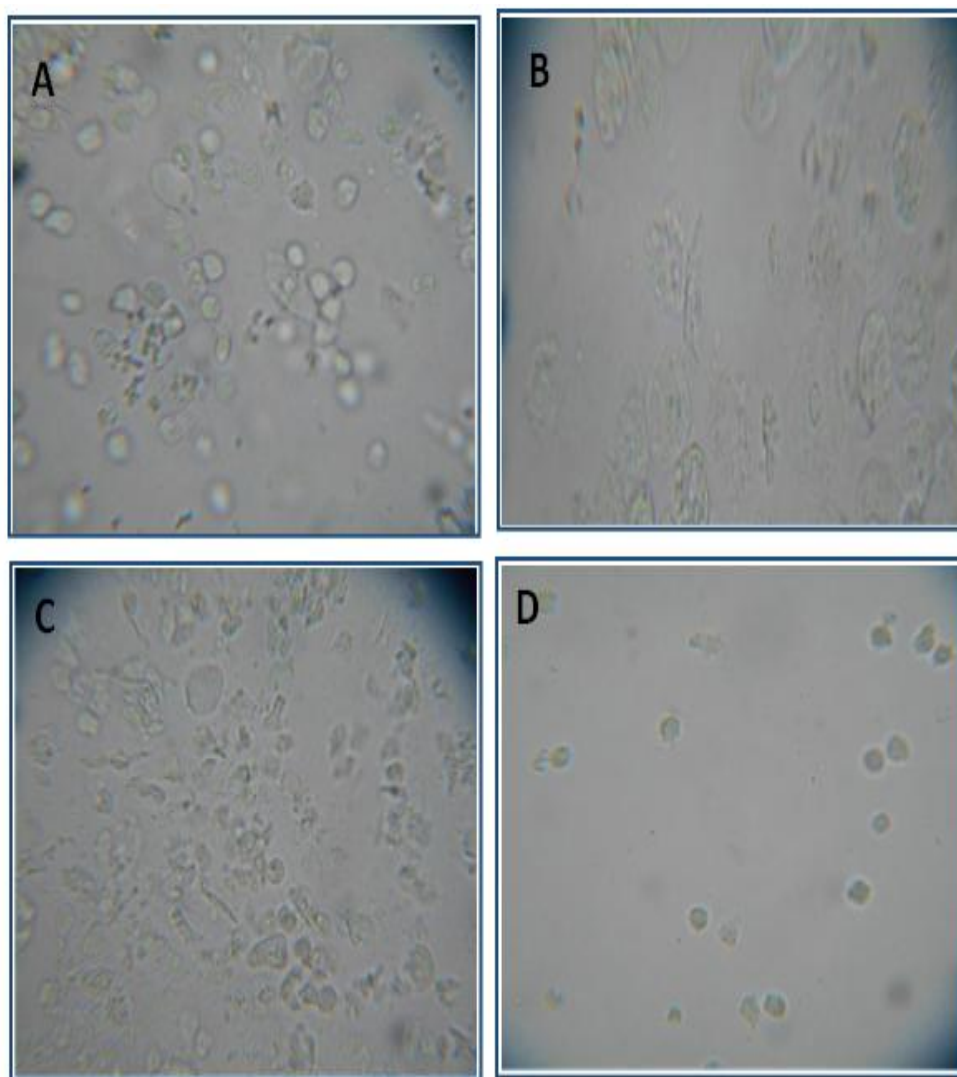
Half (50%) of the rats in the group that were administered 1 ml of 25% *Citrus aurantifolia* (Lime) juice showed regular phase sequence, P-E-M-D (Table 1). This normal pattern was observed in only one (17%) of the rats that were administered 1 ml of 50% Lime juice (Table 1). All the rats that were administered 1 ml of 100% Lime juice showed irregular patterns as none of them showed P-E-M-D sequence (Table 1).

Furthermore, analysis of the duration of the phases of animals' estrous cycles showed that the frequencies of proestrus and estrus before and after Lime juice treatment in all treatment groups were not significantly different from each other (Figures 2A and 2B). But metestrus was increased ( $p = 0.0271$ ) in rats after treatment with Lime juice (100%), when compared with the value that was obtained before treatment (Figure 2C). In addition, diestrus length was decreased ( $p = 0.0054$ ) by Lime juice (100%) treatment when the pre- and post-treatment values were compared (Figure 2D). In rats that received lower concentrations of Lime juice (25 or

50%), metestrus and diestrus values before and after treatment were not significantly ( $p > 0.05$ ) different from each other (Figures 2c and 2d). Additionally, the percentages of days in estrus and diestrus in Lime juice treatment groups were both decreased compared to pretreatment values, although this occurred mostly in rats that received 50 and 100% concentrations (Table 2). Conversely, the percentages of proestrus and metestrus phases were increased following Lime juice treatment (Table 2).

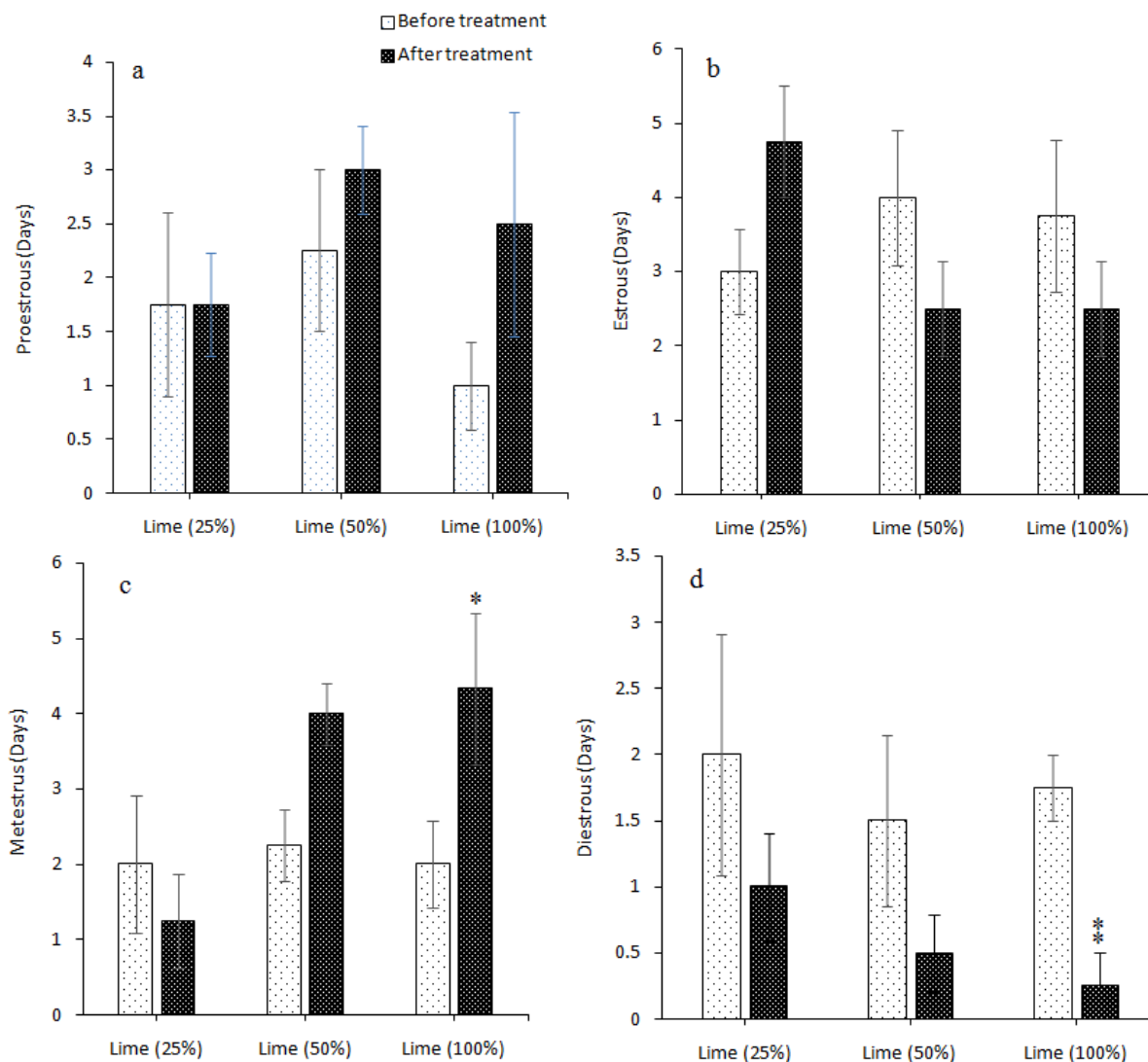
### 3.3 Effect of *Citrus aurantifolia* (Lime) Juice on Body Weight

There was an increase ( $p < 0.05$ ) in body weight in Lime juice treated rats. The weight gain in animals that received 25 or 50% Lime juice was comparable with control rats, but weight gain in those that received 100% was higher,  $p < 0.001$  (Table 3).



**Figure 1.** Photomicrographs of unstained vaginal smear from female rats (400x)

- A: Proestrus smear, characterized by predominance of nucleated epithelial cells
- B: Estrus smear, characterized by anucleated cornified cells
- C: Metestrus smear, characterized by same proportion of epithelial, cornified, and leucocyte cells
- D: Diestrus smear, primarily consisting of leucocyte cells



**Figure 2.** Fourteen days (3½ cycles) oral administration of *Citrus aurantifolia* (Lime) fruit juice prolongs metestrus and decreases diestrus phases in female Wistar rats (Animals have regular estrous cycles before Lime juice administration)

Data are expressed as mean±SEM, n=6 per group.

\* Significant at p<0.05, \*\* Significant at p<0.01.

**Table 1.** Effect of fourteen days administration of *Citrus aurantifolia* (Lime) fruit juice on estrous cycle in female rats with regular 4-day estrous cycle

Treatment	Before treatment	After treatment		
	No. of animals with regular estrous cycle phase sequence: P-E-M-D	No. of animals with regular estrous cycle phase sequence: P-E-M-D	% of animals with regular estrous cycle	% of animals with irregular estrous cycle
Lime 25% (1 ml)	6	3	50	50
Lime 50% (1 ml)	6	1	17	83
Lime 100% (1 ml)	6	0	0	100

P: Proestrus, E: Estrus, M: Metestrus, D: Diestrus

**Table 2.** Percentage of days in estrous cycle phases before and after two weeks treatment with *Citrus aurantifolia* (Lime) in female Wistar rats

Treatment group	Proestrus (%)		Estrus (%)		Metestrus (%)		Diestrus (%)	
	Before	After	Before	After	Before	After	Before	After
Lime (25%)	20.00	20.00	34.30	54.30	22.90	14.30	22.90	11.40
Lime (50%)	22.50	30.00	40.00	25.00	22.50	40.00	15.00	5.00
Lime (100%)	11.80	26.10	44.10	26.10	23.50	45.20	20.60	2.60

Animals have regular estrous cycles before Lime juice administration

**Table 3.** Effect of *Citrus aurantifolia* (Lime) fruit juice on body weight in Wistar rats

Treatment group	Body weight (g)				
	Week 0	Week 1	Week 2	Week 3	Week 4
Control	177.04±5.63	180.70±6.14	186.20±3.73*	188.10±8.65*	195.70±4.23**
Lime (25%)	181.20±6.89	189.00±7.24	191.70±5.76*	197.10±7.17*	208.10±5.09**
Lime (50%)	178.30±3.74	186.20±2.34	187.00±4.29*	187.40±3.50*	190.90±5.80*
Lime (100%)	183.90±3.92	202.50±1.70**	212.00±4.64***	211.20±3.10***	218.80±3.15***

Data are expressed as mean±SEM, n=6 per group.

\* Significant at p<0.05, \*\* Significant at p<0.01, \*\*\* Significant at p<0.0001

#### IV. DISCUSSION

Assessment of changes in vaginal epithelial cell structure in rats is routinely employed in determination of reproductive cyclicity. The rat model is also used in studies that involve reproductive toxicology because of the short length of its estrous cycle[16].From the results obtained in this study, *Citrus aurantifolia*(Lime) juice altered regular cyclicity in rats in a dose-dependent manner. Vaginal smear evaluation of rats before Lime juice treatment showed four day estrous cycles which generally followed a sequence or pattern of proestrus, estrus, metestrus, and diestrus phases. Rats with regular estrous cycles that received concentrated Lime juice (100%) all had altered phase sequence after fourteen days of treatment, whereas 83% of those that were administered 50% concentration exhibited irregular cycles. Similar observation was made in half of the animals that were given 25% Lime juice.

Further, assessmentofthe durations of estrous cyclephasesin the different animal groups showed that Lime juice does not affect proestrus and estrus at all doses, buttreatmentwith the concentrated juiceprolongsmetestrus and reduceslength of diestrus. Furthermore, the juice produced reduction in the percentage of days in estrus and diestrus, but mostly in rats that received 50 and 100% concentrations. The plant equally caused increase in percentage of days in proestrus and metestrus, thus inducing their persistence in the cycle. Percentage of days in estrus or diestrus within a treatment group over a given period of time is considered to be useful to summarize cyclicity in rats[14]. The result of the present study is thus a deviation from the normal cyclicityand can potentially affect fertility.

During the estrous cycle, prolactin, LH and FSH remain low and increase in the afternoon of the proestrus phase. Estrogen levels begin to increase at metestrus, peaks during proestrus and declines afterwards to baseline at estrus. Elevation in estrogen level at proestrus causes rapid growth of ovarian follicles[17,18], while ovulation occurs during the night of estrus 10-12 h after the LH surge[19-21].At metestrus phase, corpus luteum is formed and the uterus lining secretes progesterone, whereas at the diestrus phase, the corpus luteum now actively secretes progesterone. The period of increased progesterone secretion which is known as the luteal phase in human is known as the metestrus phase in rats. At diestrus, estrogen levels are low. The results therefore indicate that Lime juice may affect ovarian cycle and reproductive activities in rats.Reduction in percentage of estrus phase by Lime juiceas observed in this study may reduce frequency of ovulation and ultimately prevent fertility. On the other hand, shortening of diestrus phase can affect corpus luteal functionwith a possible consequence of poor fetal development, reduced number of viable offspring and fertility. This supports the earlier report of loss of pregnancies in rats following *C. aurantifolia*[9].

Furthermore, the plantenhancedanimals' body weight gain which was observed at the highest concentration. This may be due to the presence of vitamins and other vital nutritional components that may play positive roles in animals' appetite and/or provision of essential nutrients for growth.

The findings of this study are important because, though, evaluation of the human ovarian cycle may have a variety of biomarkers different from those in rats, many of the underlying endocrine mechanisms accompanied with follicular development, ovulation, pregnancy, and parturition are homologous between the

two [22]. Thus, there may be need for caution in the excessive consumption of Lime juice by females of reproductive age. However, further studies are necessary to establish these preliminary findings.

## V. CONCLUSION

From the findings, *C. aurantifolia* (Lime) fruit juice (in the undiluted form) causes changes in estrous cycle in the rat, by prolonging the duration of metestrus and concomitantly decreasing diestrus phase.

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## Conflicts of Interest

None

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