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Research Article

Citrus aurantifolia impairs fertility facilitators and indices in male albino wistar rats

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ABSTRACT

Background: The ability to reproduce is one of life's essential functions; therefore factors that affect this ability are of vital importance to mankind. We therefore designed this study to assess the effect of various dosages of *C. aurantifolia* treatment on fertility promoters and indices in male albino wistar rats.
Methods: Toxicity studies showed no lethality at 5000 mg/kg. Eighteen male albino wistar rats weighing between 220 and 240 g were used. They were randomly assigned into three groups of six rats each. Group one served as control and was gavaged 5 ml of normal saline, groups two and three were gavaged 1000 mg/kg and 1500 mg/kg body weight as medium and high dose respectively twice daily for 21 days. On the 22nd day, the rats were sacrificed and blood samples were obtained by cardiac puncture; following standard procedure, the serum was obtained for hormonal (FSH, LH, prolactin and testosterone) assay using microplate immunoenzymometric assay. The testes were harvested for semen analysis.
Results: LH level was significantly lower in medium dose ($P < 0.05$) and high dose ($P < 0.001$). A significant increase in medium dose and high dose groups ($P < 0.05$) of testosterone levels when compared to the control group was observed. There was a significant decrease in fast progressive movement and percentage normal sperm morphology of sperm cells in medium dose and high dose ($P < 0.001$). There was a significant decrease in percentage sperm concentration in medium dose ($P < 0.01$) and high dose ($P < 0.001$).
Conclusions: We therefore deduce that *Citrus aurantifolia* possess antifertility potentials in male albino wistar rats. Excessive intake should be with caution in males with fertility challenges.

Keywords: Fertility, *Citrus aurantifolia*, Semen analysis, Sex hormones, Male

INTRODUCTION

Fertility issues have been one of the major concerns of individuals, families and health-care givers. Studies have shown that more than 30% of fertility problems are male related factors.[1] Several factors are known to interfere with fertility promoters and indices such as drug treatment, environmental toxins, air pollution and stress. Probably, other factors yet to be discovered still abound.

Citrus aurantifolia belongs to the genus of flowering plants in the family Rutaceace and it is a common edible fruit of this genus. The entire lime plant has been demonstrated to have a wide range of uses including: medicinal, industrial, cosmetic, pharmaceutical etc.[2-6]

The bioactive constituents of *Citrus aurantifolia* include: Flavonoid, limonoids, Alkaloids, Ascorbic acid, Tannins.[7] Lime is readily available in every part of our society with its increasing social and medicinal uses by both men and women. For example, lime juice is used by women as a barrier contraceptive, and there is a reported history of African women douching with lime juice, lemon juice, vinegar or acidic soft drinks on the belief that it may prevent pregnancy and or sexually transmitted diseases.[8]

Parameters to assess male fertility status include: semen analysis (sperm: motility, count, morphology, volume, fracture level and pH) and hormonal assay (FSH, LH, Testosterone and prolactin). The gonadotrophs and sex steroid hormones play cardinal role in spermatogenesis and by implication, facilitation of fertility status. For instance, FSH is responsible for initiation of spermatogenesis by inducing the proliferation of spermatogonia.[9] Testosterone secretion from interstitial cells of Leydig is stimulated by Luteinizing Hormone (LH). Testosterone in turn sequences various stages of spermatogenesis.[10]

An *in vitro* study has shown that lime juice destroys both human immunodeficiency virus and sperm cells. The high acidity of the lime is suspected to be responsible for the destruction of the HIV and sperm cells.[11-14] Another study showed that lime juice reduces the numbers of ova shedded and causes irregularity in the histology of the ovaries and uterus in female rats and may possibly affect fertility.[15]

Semen analysis evaluates certain characteristics and sperm content of the semen. It is performed to help in the assessment of male fertility status, for those with procreation challenges or for the verification of the success of vasectomy. Sperm count measures the concentration of sperm cells in the ejaculate. Over 15 million sperm per millilitres is considered normal.[16] The World Health Organisation (WHO) has approved the value of 50% motility measured within 60 seconds of collection as normal. The WHO criteria for normal morphology as described in 2010 state that a sample is normal if 4% (or 5th percentile) or more of the observed sperm have normal morphology.[16] Semen volumes between 1.0-6.5ml are said to be normal.[17]

It has been reported that oligospermic or azoospermic patients with normal gonadotropins show relatively higher serum levels of prolactin, proving a role of prolactin in gametogenesis.[18,19] There are many studies suggesting that hyperprolactinaemia has a definite role in the male infertility and is one of the reversible causes of infertility.[19,20]

In view of the foregoing, this study was therefore designed and conducted with the overall objective to assess the effect of various dosages of *C. aurantifolia* treatment on fertility promoters and indices in male albino wistar rats. The result of this study, we believe will provide further scientific information on the effect of consumption of *C. aurantifolia* by extrapolation in humans.

METHODS

Plant material

Fresh lime fruits were obtained from an early morning market in Uyo metropolis and were identified at the department of botany and ecological studies of the University of Uyo, Nigeria. The fruits were carefully washed to remove sand and dirt and sliced into two halves and was gently squeezed into a container. The obtained lime juice was filtered through a filter paper; the seeds and residual pulp were discarded. Fifty four lime fruits were processed in like manner, collected into a clean plastic vessel, covered and stored in the refrigerator. The pH of the lime juice was 1.7. A preliminary study was conducted on lethal dose (L_{D50}), and no lethality was recorded at 5000 mg/kg.

Animal preparation, experimental groupings and treatment

Eighteen male albino wistar rats were used for this study. The animals were randomly assigned to three groups such that each group had six (6) animals. Group 1 served as the control group fed with normal rat chow (feed). Group 2 served as the medium dose group and were given 1000 mg/kg glucose orally, twice daily. Group 3 served as the high dose group and received 1500 mg/kg *Citrus aurantifolia* orally, twice daily. All animals had access to water ad libitum. The cages were well ventilated, exposed to normal temperature and 12/12 hours light/dark cycle. After fourteen days of acclimatization, oral administration of lime juice to groups 2 and 3 commenced. The animals were sacrificed after 21 days. All experiments were examined and approved by the ethical committee of the University of Uyo on animal research and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Sperm cell concentration

Testes were crushed into pieces, diluted in 5 ml of normal saline and allowed for 5-10 minutes to enable the spermatozoa to spread out into the diluents solution. 1 ml of supernatant was diluted in 100 ml solution. 0.01 of the suspension was loaded into a charged Neubauer counting chamber and cover slipped. It was allowed to rest for 10 minutes and observed microscopically. Number of cells were counted in millions/ml.

Sperm morphology

1 ml of seminal fluid was diluted with 20 ml of buffered formol saline and then 0.01ml of the solution was loaded on a grease free slide with cover slip and viewed under a microscope and the following were observed: tail defect, neck defect, mid-piece defect, head defect and percentage of normal morphology was determined.

Sperm motility

1 ml of seminal fluid was diluted with 20mls of buffered formol saline and 0.01 ml of the solution was loaded on a grease free slide and covered with a cover slip and observed microscopically.

Serum FSH, LH and prolactin measurements

The FSH, LH and prolactin were determined based on the principle of sandwich method. The assay system utilizes a high affinity and specificity monoclonal antibody (enzyme conjugated and immobilized) directed against a distinct antigenic determinant on the intact FSH, LH and prolactin molecule. The test sample is allowed to react simultaneously with two antibodies, resulting in the FSH, LH and prolactin molecules being sandwiched between the solid phase and enzyme - linked antibodies.

After incubation, the wells were washed with washing solution to remove unburned labeled antibodies. Tetra methyl benzidine substrate is added and incubated, resulting in the development of a blue colour. The colour development is stopped with addition of stopping reagent, changing the colour to yellow. The concentration of FSH, LH and prolactin is directly proportional to the colour intensity of the test sample.

Serum testosterone measurement

Testosterone level is determined using competitive microplate enzyme immunoassay. Plates are coated with anti-testosterone antibodies. Calibrator specimen is first added to microplate well. Enzyme testosterone conjugate is added. Testosterone present in the sample competes with enzyme-testosterone conjugate for building with anti-testosterone counted microplate to form an antigen-antibody complex. Unbound conjugate is removed by washing. The enzyme activity in the antibody-bound fraction is inversely proportional to the native testosterone concentration. The enzyme activity is revealed by colour change in tetramethylbenzidine substrate solution.

Statistical analysis

All results were presented as mean ± standard error of mean. Three sets of data were analyzed using one way ANOVA, followed by the Least Significant Difference (LSD) procedure for significant F values, (P = 0.05) was considered significant. Computer software SPSS and excel analyzer was used for the analysis.

RESULTS

Comparison of FSH levels in different experimental group treated with Citrus aurantifolia

Mean values of FSH level in control medium dose and high dose were mean ± SEM, 0.12 ± 0.02, 0.02 ± 0.02 and 0.13 and 0.13 ± 0.2 respectively. There was no significant difference in FSH level, though a marginal increase was observed in the high dose (Figure 1).

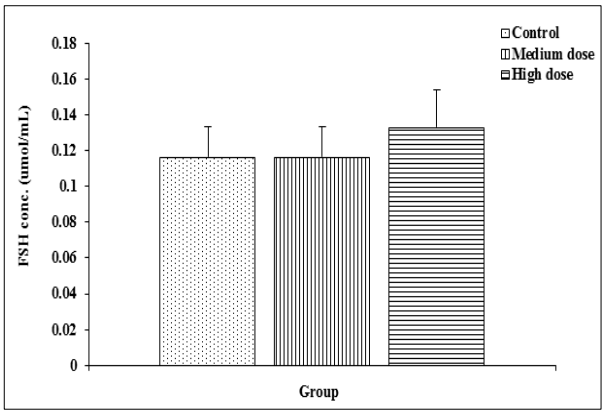

Figure 1: Comparison of follicle stimulating hormone (FSH) levels in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6

Comparison of LH levels in different experimental group treated with Citrus aurantifolia.

Mean values of LH level in control medium dose and high dose were mean ± SEM, 0.18 ± 0.02, 0.12 ± 0.02 and 0.10 ± 0.00 respectively. LH level was significantly lower in medium dose (P <0.05) and High dose (P <0.001) when compared to the control group (Figure 2).

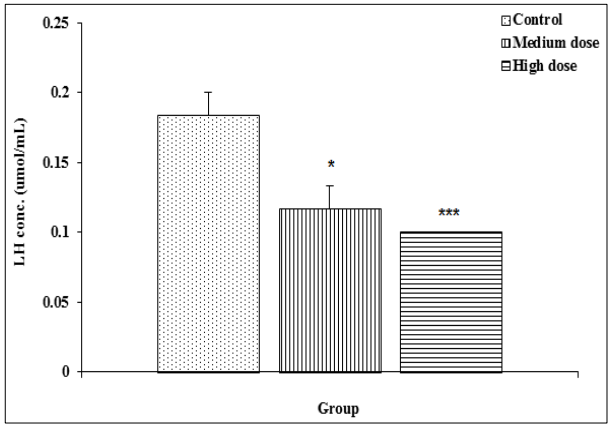

Figure 2: Comparison of luteinizing hormone (LH) levels in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6
*P <0.05, ***P <0.001 vs. control

Comparison of prolactin levels in different experimental group treated with Citrus aurantifolia

Mean values of LH level in control, medium dose and high dose were mean ± SEM, 0.12 ± 0.02, 0.10 ± 0.00 and 0.10 ± 0.00 respectively. There was no significant difference in prolactin levels (Figure 3).

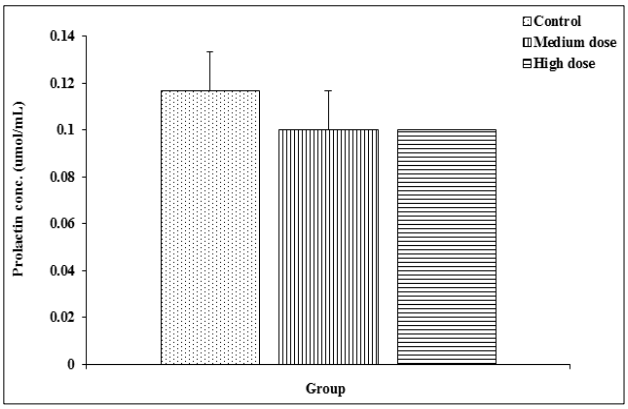

Figure 3: Comparison of prolactin levels in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6

Comparison of testosterone levels in different experimental group treated with Citrus aurantifolia

Mean values of LH level in control medium dose and high dose were mean ± SEM, 8.12 ± 2.01, 28.63 ± 7.81 and 22.58 ± 58 ± 5.21 respectively. There was significant increase in medium dose and high dose groups (P <0.05) when compared to the control group (Figure 4).

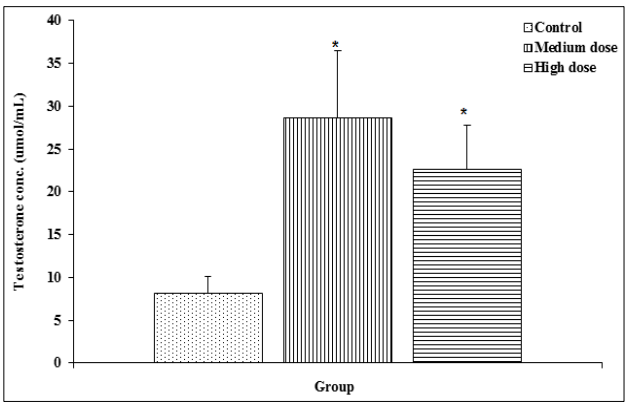

Figure 4: Comparison of testosterone levels in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6
*P <0.05 vs. control

Comparison of fast progressive movements (sperm motility) in different experimental group treated with Citrus aurantifolia

Mean values of fast progressive movements (sperm motility) in control, medium dose and high dose groups were, mean ± SEM: 71.67 ± 2.79, 55.00 ± 1.83 and 54.33 ± 1.48 respectively. There was a significant decrease in fast progressive movement of sperm cells in medium dose and high dose (P <0.001) when compared to the control group (Figure 5).

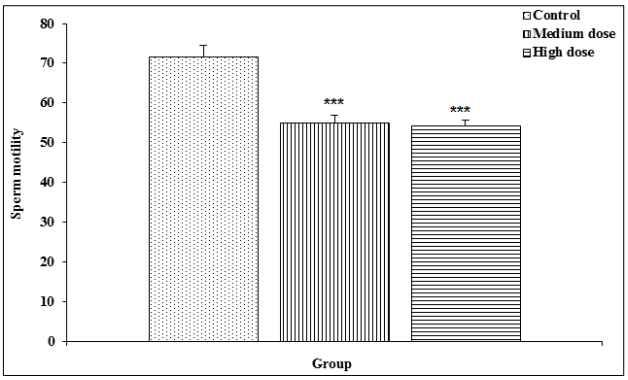

Figure 5: Comparison of fast progressive movement of sperm in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6
***P <0.001 vs. control

Comparison of percentage sperm concentration in different experimental groups treated with Citrus aurantifolia.

Mean values of percentage sperm concentration in control, medium dose and high dose groups were, mean ± SEM: 69.67 ± 2.08, 61.33 ± 1.52 and 45.67 ± 0.42 respectively. There was a significant decrease in percentage sperm concentration in medium dose (p<0.01) and high dose (P <0.001) when compared to the control group. There was a significant decrease in percentage sperm concentration in the high dose (P <0.001) when compared to the medium dose (Figure 6).

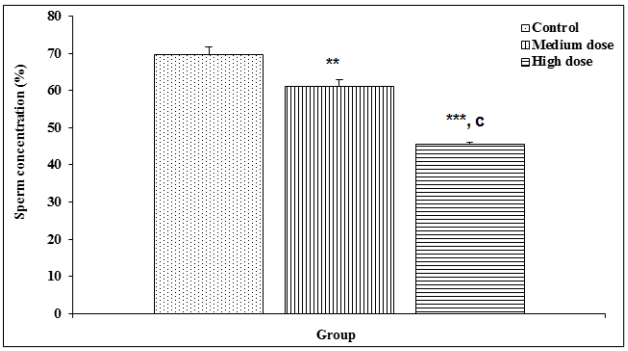

Figure 6: Comparison of percentage sperm concentration in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6
**P <0.01, ***P <0.001 vs. control; c = P <0.001 vs. medium dose

Comparison of percentage normal sperm morphology in different experimental groups treated with Citrus aurantifolia.

Mean values of percentage normal sperm morphology in control, medium dose and high dose groups were mean ±

SEM: 81.33 ± 0.56, 43.00 ± 0.37 and 54.67 ± 0.92 respectively. There was significant decrease in percentage normal sperm morphology in medium dose and high dose (P <0.001) when compared to the control group (Figure 7).

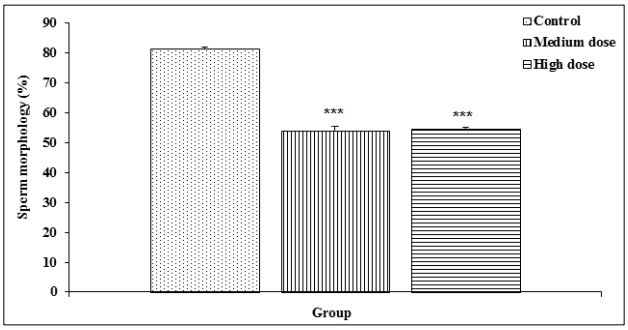

Figure 7: Comparison of percentage normal sperm morphology in different experimental groups treated with *Citrus aurantifolia*.

Values are expressed as mean ± SEM, n = 6
***p<0.001 vs. control

DISCUSSION

Report from previous research study had indicated the anti-fertility effect of *Citrus aurantifolia* on estrous cycle and ovulation of Sprague-Dawley rats.[21] The result from our study has further strengthened the need for caution in the indiscriminate consumption of lime juice especially in subject with fertility expectancy. Our findings assert the findings that *Citrus aurantifolia* does not only affect female rats reproductive process as previously reported but the male as well. This study has clearly demonstrated that *Citrus aurantifolia* is a potent inhibitor of fertility promoters and indices in male albino wistar rats. The result showed a dose dependent decrease in LH level and sperm concentration. Fast progressive movement of sperm and percentage normal sperm morphology were also significantly decrease by treatment with *Citrus aurantifolia* juice. There were no significant changes for FSH and Prolactin. However, testosterone showed a significant increase with both medium and high dosage administration of *Citrus aurantifolia*.

Sperm concentration, motility and viability are crucial indices that determine the potential of sperm to fertilize an ovum. Poor semen characteristic has been linked to damage spermatocyte DNA.[22,23] It has also been reported that the integrity of spermatocyte DNA is protected by zinc and citric acid secreted from the prostate gland.[24] The mechanism involves neutralization of reactive oxygen species. It is therefore most likely that certain phytochemical constituents of *Citrus aurantifolia* juice might have compromise either the normal secretion of zinc and citric acid by the prostate or interrupt an enzymatic pathway involve in reactive oxygen species deactivation. Hence, the resultant decreases in sperm quantity and quality.

In the male, an increase in prolactin level can cause a decrease in LH and testosterone levels resulting in low sperm concentration. LH is known to stimulate the interstitial cells of Leydig to synthesis and secrete testosterone. However, in this study, prolactin showed no significant changes though a marginal decrease was observed. This implies that, the decreased level of LH was not related to prolactin effect. The low LH concentration may therefore be due to the negative feedback signal triggered by rising testosterone levels as was the case in this study.

Present in *Citrus aurantifolia* as in most plants are biologically active macromolecules such as alkaloids, saponins, flavonoids, tannins, limonoids etc. How these active constituents selectively or synergistically contribute to the antifertility effect of lime juice is still a subject of interest for further research. Flavonoids in lime juice have been reported to exert mild anti-oxidative effects in lipid peroxidation.[25] Limonoids are highly oxygenated modified triterpenes derived from a precursor with 4, 4, 8-trimethyl-17 furanyl steroid skeleton.[26] Increasing body of evidence seems to suggest that limonoids and flavonoids have independent biological activities.[27] Alkaloids are also major component of stimulant agents like cocaine, caffeine and nicotine. Studies have shown that exposure of human sperm to nicotine *in vitro* decreases the percentage of motile sperm.[28] The alkaloid constituent has been implicated as responsible for this effect. As obtained from our result, the decrease in the percentage sperm motility in this study strongly supports these findings because *Citrus aurantifolia* is a rich source of alkaloids.

In an *in vitro* study, it was reported that about 2000 sperm cells were destroyed within 30 seconds of applying a quantity of lime juice and it was postulated that high acidity might have been responsible. Though we did not measure the pH of the lime juice administered in this study, previous study has earlier confirmed the low pH of lime juice.[29] There was a dose dependent reduction in sperm motility, percentage sperm concentration and percentage normal morphology all pointing to the anti-fertility potency of lime juice, which is directly or indirectly related to its physico-chemical characteristics and phytoconstituent.

Findings from this study strongly demonstrate the antifertility potential of *Citrus aurantifolia* in male albino wistar rats. Almost all fertility promoters and indices including hormones and sperm quality and quantity were compromised. Excessive consumption should therefore be discouraged in males with fertility compromise.

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Conflict of interest: None declared
Ethical approval: The study was approved by the University of Uyo Animal Research Ethics Committee in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki

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