

# Anti-fertility and Sexual Behavioral Effects of Sub-Acute Administration of *Carpolobia Alba* (G. Don) Root Extract in Male Wistar Rats: an Immunohistochemical Study

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## Research Article

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# Abstract

## Background:

Over 80% of the population in Africa and Asia rely on traditional medicine as their primary form of health care. For centuries, plant-based aphrodisiacs have been used to enhance sexual performance. In sub-Saharan Africa, especially Nigeria, *Carpolobia alba* (G. Don) is widely used for its perceived sexual stimulatory effects, despite limited scientific evidence on its impact on male fertility. In this study, we investigated the effects of sub-acute administration of methanol root extract of *Carpolobia alba* on sexual behaviour and testicular immunoexpression of B-Cell lymphoma-2 (Bcl-2) and Proliferating Cell Nuclear Antigen (PCNA) in adult male Wistar rats.

## Methods:

Twenty adult male rats (weighing 200g-250g) were randomly divided into four groups consisting of ten animals each. Each group was treated daily with distilled water, 100, 200 and 400mg/kg body weight respectively of methanol root extract *Carpolobia alba* G.Don for 30 days. The animals were anesthetized using Isoflurane via an induction chamber. Mating behavioral assessment was done by direct observation and use of camera, testicular B-Cell lymphoma-2 (Bcl-2) and Proliferating Cell Nuclear Antigen (PCNA) Immunoexpression was done using immunohistochemical techniques.

## Results:

Our result shows significantly ( $P < 0.05$ ) increased sexual activity and mating behavior across treated groups. However, testicular sections revealed a weak immunoexpression of Bcl-2, indicating increased apoptotic activity, and strong PCNA expression, indicating increased cell proliferation.

## Conclusion:

Despite its traditional use as an aphrodisiac, *Carpolobia alba* exhibits potential anti-fertility effects following sub-acute exposure, as evidenced by disrupted testicular cell regulation and structural integrity. These findings raise important concerns about its long-term reproductive safety and need for further investigation.

## INTRODUCTION

The desire to increase sexual arousal, pleasure and potency has existed since the beginning of life. Sexual response in male and female reflects a dynamic balance between excitatory and inhibitory signals of the autonomic nervous system within the external genitalia and throughout the central nervous system (Goldstein, 2022). An aphrodisiac is any natural chemical or agent that enhances sexual

desire, attractiveness, pleasure, behavior, and performance (Sandroni, 2001). It can be derived from animal, plant, spices or mineral (Abdullahi and Tukur 2013). Aphrodisiac chemicals are overused and recreationally used for sexual enhancement, even though their primary indication is erectile dysfunction (Christopher and Cindy, 2011).

The rate of consumption of natural aphrodisiacs especially among young men has increased in recent times. Furthermore, there is limited scientific evidence supporting the efficacy and safety of natural aphrodisiacs which is worrisome as it could lead to abuse of aphrodisiacs (Manortey et al, 2018). A common aphrodisiac traditionally used in Nigeria is *Carpolobia alba*. *Carpolobia alba* grows as a shrub or small tree which is 3 to 6 metres (9.8 to 19.7 ft) tall. It has querulous branches or short pubescent. Its leaves are membranous or slightly leathery. This type of plant belongs to the milkwort family (Polygalaceae) and is native to Western Tropical and Central Africa's rainforests, forest edges, and savanna parks at elevations lower than 400 meters (1,300 feet). *Carpolobia alba* is a popular natural recreational aphrodisiac plants commonly used by pygmies of the forest region (Ipassa of Garbon, South-Cameroon; and the Yoruba, Efik, Ibo, and Ijaw peoples of Nigeria, ) and other part of sub-Sahara Africa (Nwidu et al., 2015). It is commonly called Cattle stick in English, *Egbo Oshunshun* in Yoruba, *Angalagala* in Igbo, *Ikpafum* in Ibibio (Nwidu et al., 2015). The root of *Carpolobia alba* has long been used to treat male infertility, increase virility, and improves male fertility. It has been reported to have aphrodisiac and androgenic effects. (Manfo et al., 2014).

The apoptotic pathway in the mitochondria is controlled by proteins from the BCL-2 family, which controls cell survival and death (Czabotar and Garcia-Saez, 2023). The BCL-2 is located in the outer membrane of the mitochondria and maintains cell survival by counteracting the effects of the proapoptotic protein BAX, thereby preserving mitochondrial membrane integrity. (Morris et al., 2021). BCL-2 plays a crucial role in regulating apoptosis. The expression of BCL2 is tightly controlled and disturbance of BCL2 has been implicated in various disease conditions including cancer (Adelakun et al., 2024).

Proliferating Cell Nuclear Antigen (PCNA) is a marker for cell proliferation and is involved in DNA synthesis, DNA methylation, chromosome recombination, RNA transcription and nucleic acid metabolism (Zhao et al., 2018). Therefore, changes in PCNA expression are used as a marker to evaluate cell proliferation. Spermatogenesis a complex process by which spermatogonia develops into mature sperm cell. It involves continuous cell division and proliferation. Thus, PCNA expression levels in the testis is a standard marker of spermatogenic cell proliferation and can be used also to assess the state of spermatogenic cell proliferation (Farang *et al.*, 2021). Apoptosis is accompanied by proliferation. Therefore, the positive expression of PCNA indicates increased proliferation of spermatogenic cells in response to apoptotic cell death. A study carried out by Zhao et al. (2018) reported that positive expression of PCNA in the testes correlates with spermatogenesis dysfunction in fluoride-treated rat. The aim of this study is to investigate the effects of sub-acute administration of Methanol root Extract of *Carpolobia alba* (G. Don) on the sexual behavior and the level of expression of BCL-2 and PCNA in the testicular tissue of male Wistar rat.

# MATERIALS AND METHOD

## Plant Harvest and Extraction

The *Carpolobia alba* root was obtained from Ayede Ogbese in Akure North Local Government, Ondo state, Nigeria and it was authenticated in the herbarium of Department of Plant Biology and Biotechnology, University of Medical Sciences Ondo state, Nigeria with voucher number UNIMED P.B.T.H No 034. After air drying, the *Carpolobia alba* root (2.20kg) was pulverized into powder after which *Carpolobia alba* root was extracted using Soxhlet with pure methanol as the solvent. After filtering the methanol containing the extract, the solvent was vacuum-distilled in a rotary evaporator at 4°C. To verify that all remaining solvent was gone, the remaining extract was lastly dried for two hours at 30°C in a vacuum oven. 90.22g (1.69% yields) of the powdery mass were obtained which was stored at room temperature for the study. Distilled water was used to prepare a fresh solution of the extract.

## ANIMAL EXPERIMENTATION AND DESIGN

Twenty male Wistar rats (weighing 200-250g) were procured from the Animal facility of University of Medical Sciences, Ondo State, Nigeria. The rats were acclimatized for two weeks before the experiment begins. The experimental animals were accommodated in standard laboratory conditions. The animals were exposed to light and dark cycle of 12 hours and fed rat show and water *ad libitum*. Twenty adult Wistar rats were randomized into four groups consisting of five rats each. Group 1 (control) received only distilled water; Group 2,3 and 4 received 100mg/kg, 200mg/kg and 400mg/kg body weight respectively of methanol root extract of *Carpolobia alba* for 30 days.

### Assessment of Mating behaviour parameters

The primed female rats that exhibited profound receptivity were introduced into the cages containing male rat in ratio 1:1 after which the following mating behavior were observed directly from the cage sides and via camera for 30 minutes (Yakubu and Jimoh, 2014): The following mating behavioural parameters were assessed: Mount frequency (MF) (number of mounts with or without intromission from the time of introduction of the female until ejaculation); Intromission frequency (IF) (number of vaginal penetrations during the observation period); Ejaculatory frequency (EF) (number of ejaculatory recorded during the time of observation); Mount latency (ML) (time interval between the introduction of the female and the first mount by the male); Ejaculation latency (EL) (time interval between the first intromission and ejaculatory); Intromission latency (IL) (time interval between the introduction of the female and the first intromission by the male). The assessment was done around 1:30pm local time to avoid interference of with the natural increase in libido in male rat in the morning (Kenmogne *et al.*, 2016).

## IMMUNOHISTOCHEMISTRY ASSAY

Paraffin embedded sections where deparaffinized with xylene, and rehydrated through descending grades of ethanol (100%, 95%, 70 % ethanol) and taken to water. Heat-mediated antigen retrieval was

performed using a citrate-based antigen unmasking solution, pH 6.0 (Vector<sup>®</sup>, Burlingame, CA, USA; #H3300) in a steamer for 30 mins. Thereafter, it was Wash sections in PBS for 2 minutes. Endogenous peroxidase blocking in 0.3 % hydrogen peroxide solution in PBS for 10 minutes and Wash sections in PBS for 2 minutes. Sections were then incubated at room temperature for 2 hours in primary rabbit antibodies diluted in a universal antibody diluent and blocking reagent, UltraCruz<sup>®</sup> Blocking Reagent (Santa Cruz, USA). The sections were subsequently incubated with specific primary antibodies against PCNA (Cell Signalling, USA; #13110) and Bcl-2 (Novus Biologicals, USA; NB100-56098) overnight at 4 °C in a humidified chamber. Section was washed in PBS for 5 minutes, incubated in ImmPRESS<sup>™</sup> HRP Anti-Rabbit IgG (Peroxidase) Polymer Reagent, made in horse for 30 mins, Wash in PBS for 5 minutes twice. Colour is developed with DAB Peroxidase (HRP) Substrate Kit (Vector<sup>®</sup> Labs, USA). Sections were rinsed well in tap water. Sections were counter-stained in hematoxylin and observed under a microscope.

### **Quantification of Immunoexpression**

The expressions for BCL-2 and PCNA in the testicular tissue were quantified using Image J software version 1.54p. The data obtained were statistically analyzed.

### **Statistical Analysis**

The analysis was done with GraphPad Prism software version 10.1. Both descriptive and inferential statistics (one-way ANOVA) were done on all obtained data. Turkey's multiple comparison was used to test for statistical significant difference between control and experimental groups. The data were represented as Standard Error of Mean and the level of differences were considered significant at  $p < 0.05$ .

### **Ethical Consideration**

Ethical approval was obtained from Olabisi Onabanjo University's research Ethic Committee with reference number: **OOU/SCIENG/EC/0009/240924**.

## **RESULT**

### **Assessment of mating behavior**

The mounting (MF), Intromission (IF), and Ejaculation (EF) frequencies were significantly increased ( $P < 0.05$ ) in the experimental groups when compared to the control (fig 1,2 and 3). This means that animals that received the extract had more sexual motivation, penile thrusting and sexual climax than the control group. The mounting latency (fig 4) and intromission latency (fig 5) was significantly reduced ( $P < 0.05$ ) in the experimental group when compared to control. This mean the animals that received the extract took lesser time to make their first mount and penile thrust after the introduction of a receptive female rat. On the contrary, the ejaculation latency (fig 6) was significantly increased in the experimental group when

compared to the control. This implies that animals that receive the extract took longer time before ejaculation during mating.

IHC Assay of BCL-2 Expression in the Testicular Tissue

IHC Assay of PCNA Expression in the Testicular Tissue

## DISCUSSION

In this study the effects of sub-acute administration of Methanol root Extract of *Carpolobia alba* (G. Don) on mating behavior and testicular PCNA and BCL-2 immunoexpression in male Wistar rat was investigated. The result obtained from the mating behavioral assessments shows that there is significant increase in mounting frequency (MF), intromission frequency (IF) and ejaculation frequency (EF) in all the experimental groups (100,200 and 400mg/kg groups) when compared to control. An increase in MF, IF, and EF reflects enhanced sexual motivation. This implies that *Carpolobia alba* increases sexual motivation of the male rats to make with the female rat. This result agrees with study reported by (Kenmogne et al., 2016) who reported that aqueous extract of *Carpolobia alba* significantly increase mount frequency. This suggests that *Carpolobia alba* significantly increase sexual drive in male Wistar rat. The mounting and intromission latencies were significantly reduced in the experimental groups compared to the control. This mean the animals that received the extract took lesser time to make their first mount and vaginal thrust after the introduction of a receptive female rat. The reduction in mount and intromission latencies may also connote increased orientation activities towards the receptive females and stronger sexual motivation (Yakubu and Jimoh, 2015). On the contrary, the ejaculation latency (Fig. 6) was significantly increased in the experimental group when compared to the control. The prolonged EL in *Carpolobia alba* treated group suggests that the extract enhanced sexual intercourse for sufficiently desired time. This could also suggest the activation of nitric oxide and regulating the release and serotonin.

B-cell lymphoma 2 (BCL2) is an anti-apoptotic protein that is important and crucial in regulating programmed cell death (apoptosis). It functions by inhibiting the activity of pro-apoptotic proteins, thereby promoting cell survival. The result from this study shows that experimental groups treated with *Carpolobia alba* exhibited weak expression of BCL2 compared to the control group. This suggests that the plant extract may have a pro-apoptotic effect, potentially leading to increased cell death in the testes. The Bcl-2 immunoexpression were reduced in the experimental group but the reduction was only significant at 400mg/kg which suggest a dose dependent effects. This indicates that the pro-apoptotic effects of the extract which may be exacerbated by higher doses. This result agrees with the study reported by Sarica *et al* (2001) who reported bcl2 downregulation in apoptotic cells.

Proliferating Cell Nuclear Antigen (PCNA) is a critical marker for cell proliferation and is involved in DNA replication and repair. PCNA protein expression serve as a biomarker of proliferation of spermatogenic cells and can be used to assess the proliferation state of spermatogenic cells. The result from this study showed significantly increased PCNA expression (at 100 and 400mg/kg) in the *Carpolobia alba*-treated

group compared to the control group. This suggests enhanced spermatogenic activity in response to the pro-apoptotic effects of extract. Apoptosis is accompanied by proliferation. During spermatogenic cell mitosis and differentiation, PCNA as a cell proliferation factor is involved. PCNA not only regulate cell proliferation but also act as cell proliferation markers. This result is consistent with the work reported by Zhao et al. (2018) who documented positive immunoexpression of PCNA in fluoride-induced testicular toxicity. Also, similar result was documented by Oluwatunase et al. (2024) who reported that sub-acute treatment of *Carpolobia alba* induces apoptotic spermatogenic cell death, oxidative stress and inhibits synthesis of reproductive hormones.

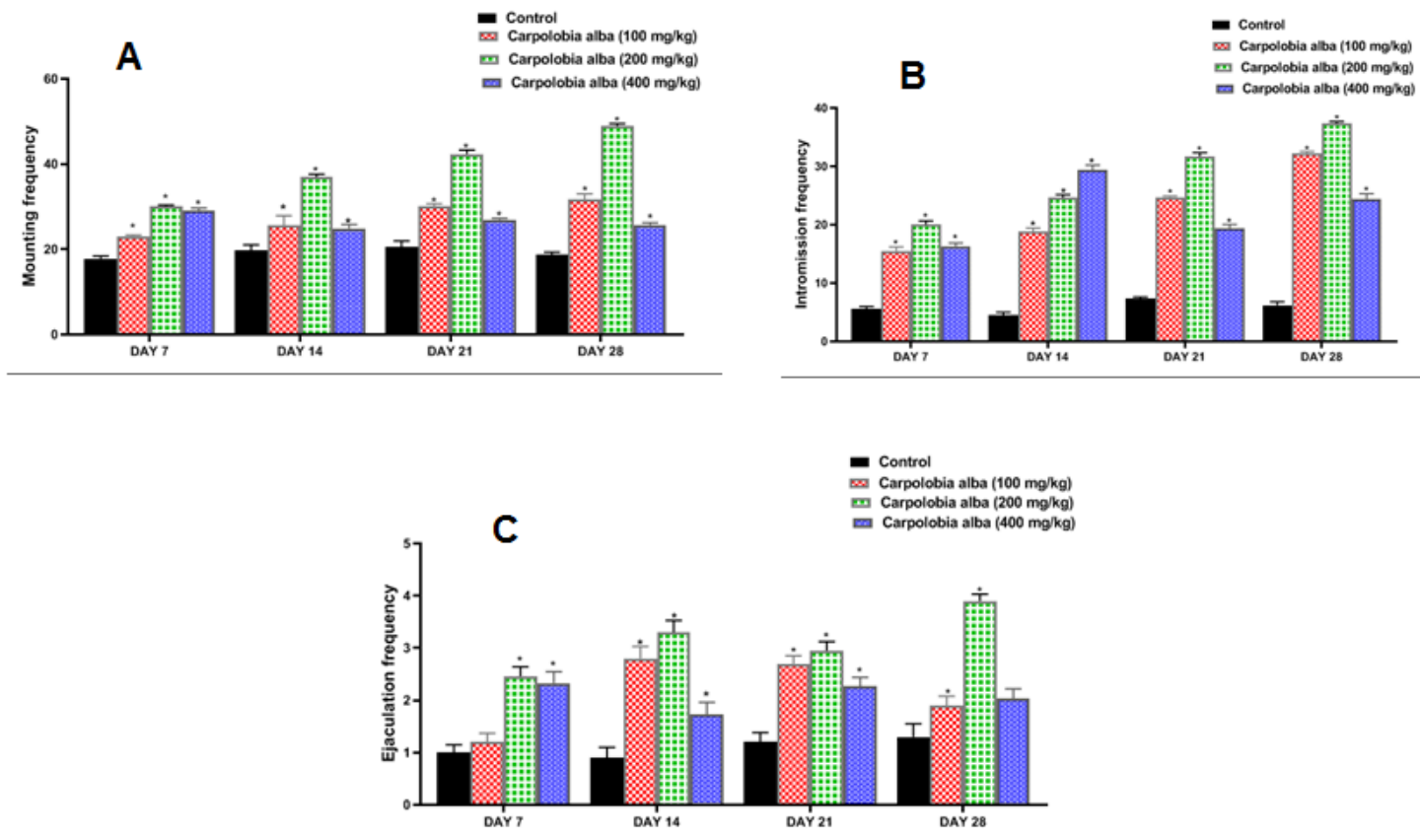
In conclusion, sub-acute administration of methanol root Extract of *Carpolobia alba* (G. Don) improves sexual behavior and induces spermatogenic apoptotic cell death. This implies that while the extract enhances sexual desire, motivation and activities, it could have deleterious effects on the spermatogenic cells which lead to infertility.

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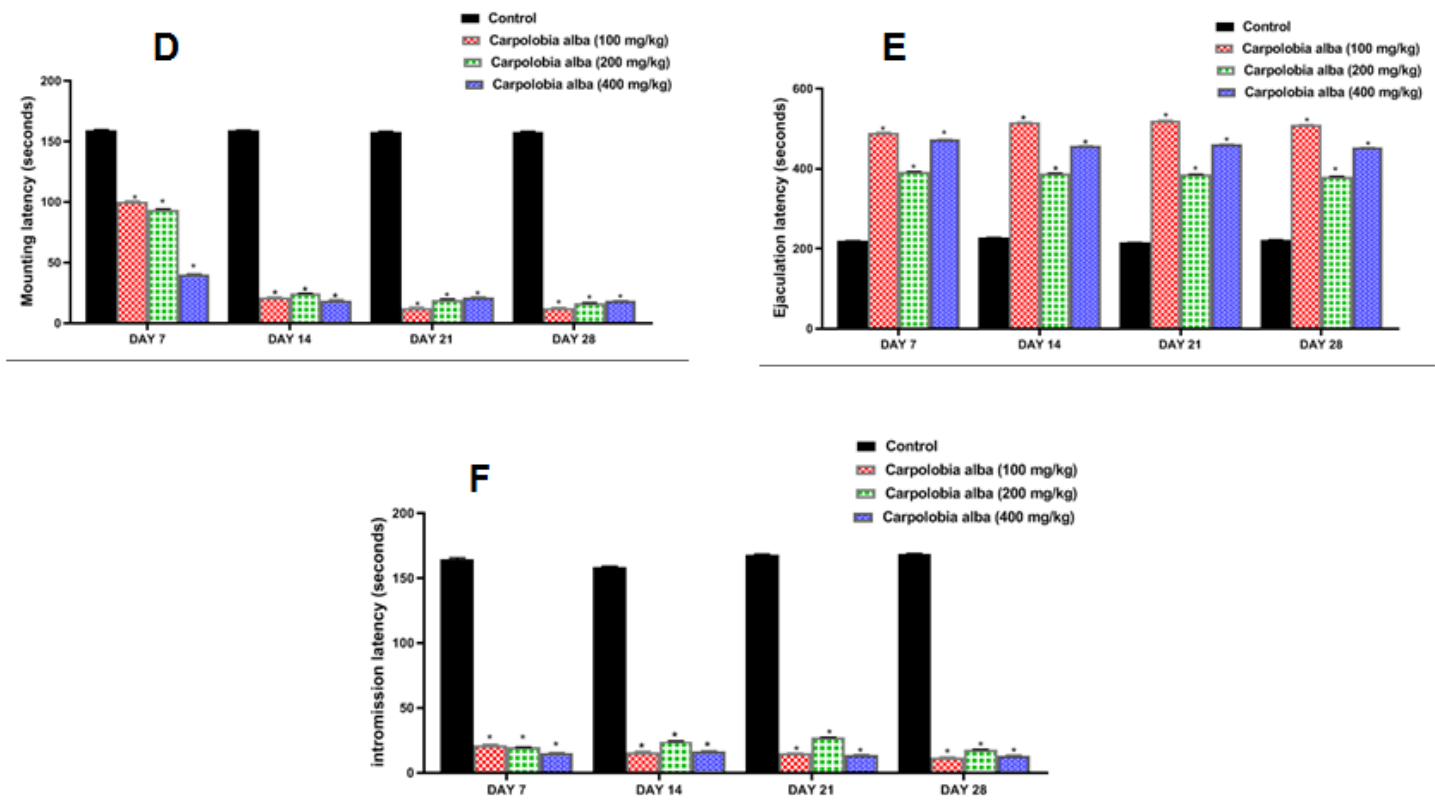
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## Figures



**Figure 1**

Effects of administering *Carpolobia alba* root methanol extract for 30 days on the mounting frequency (A), Intromission frequency (B) and Ejaculation frequency (C) of male rat. Bars represent Mean  $\pm$  SEM (n=5) (one-way ANOVA followed by Tukey *post hoc test*). \*p< 0.05 relative to control.



**Figure 2**

Effects of administering *Carpolobia alba* root methanol extract for 30 days on the mounting latency (D), ejaculation latency (E), intromission latency (F) of male rat. Bars represent Mean  $\pm$  SEM (n=5) (one-way ANOVA followed by Tukey *post hoc test*). \* $p < 0.05$  relative to control.

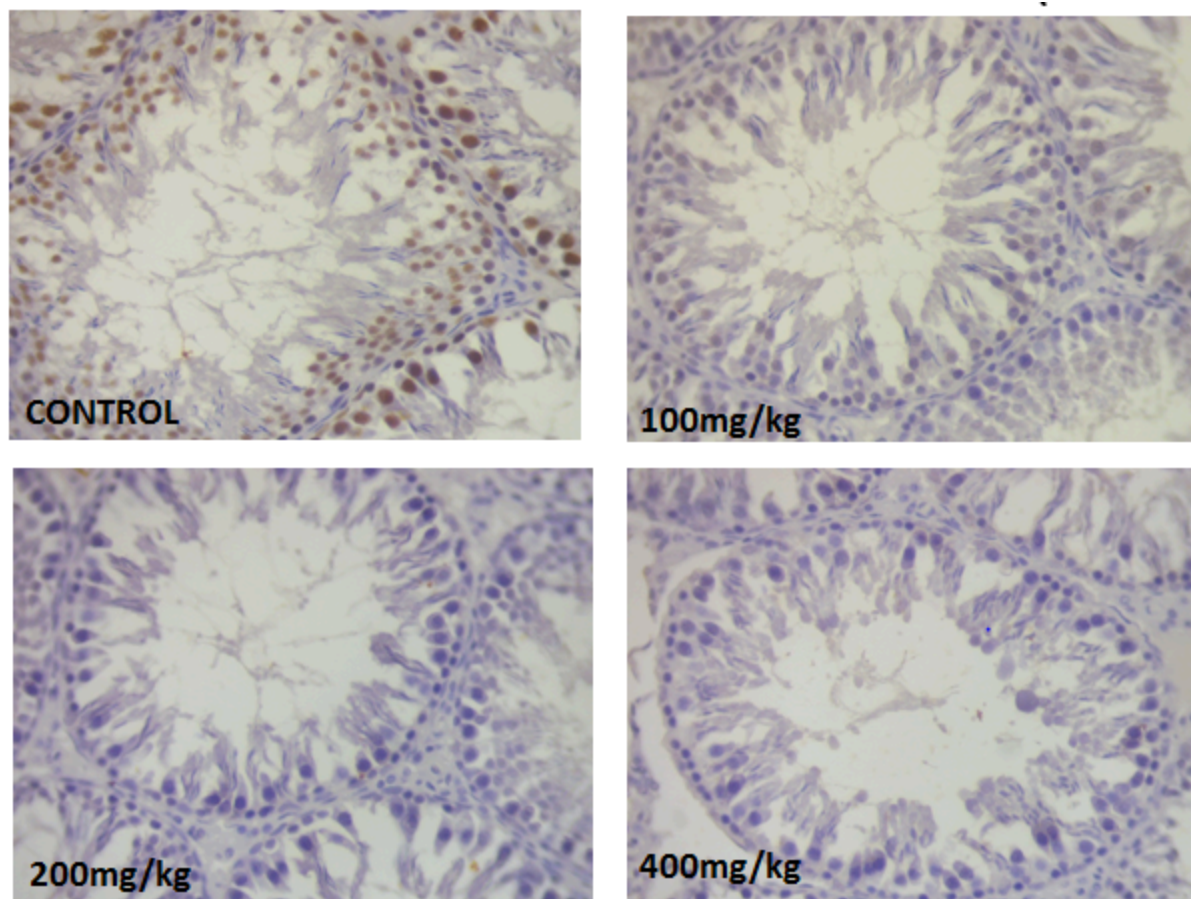
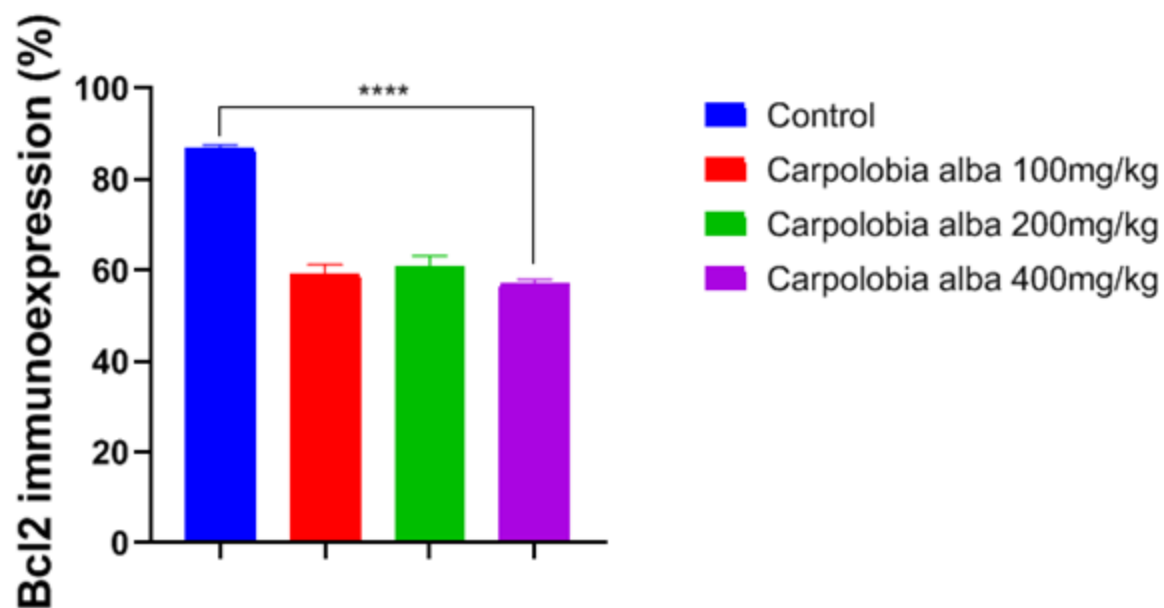


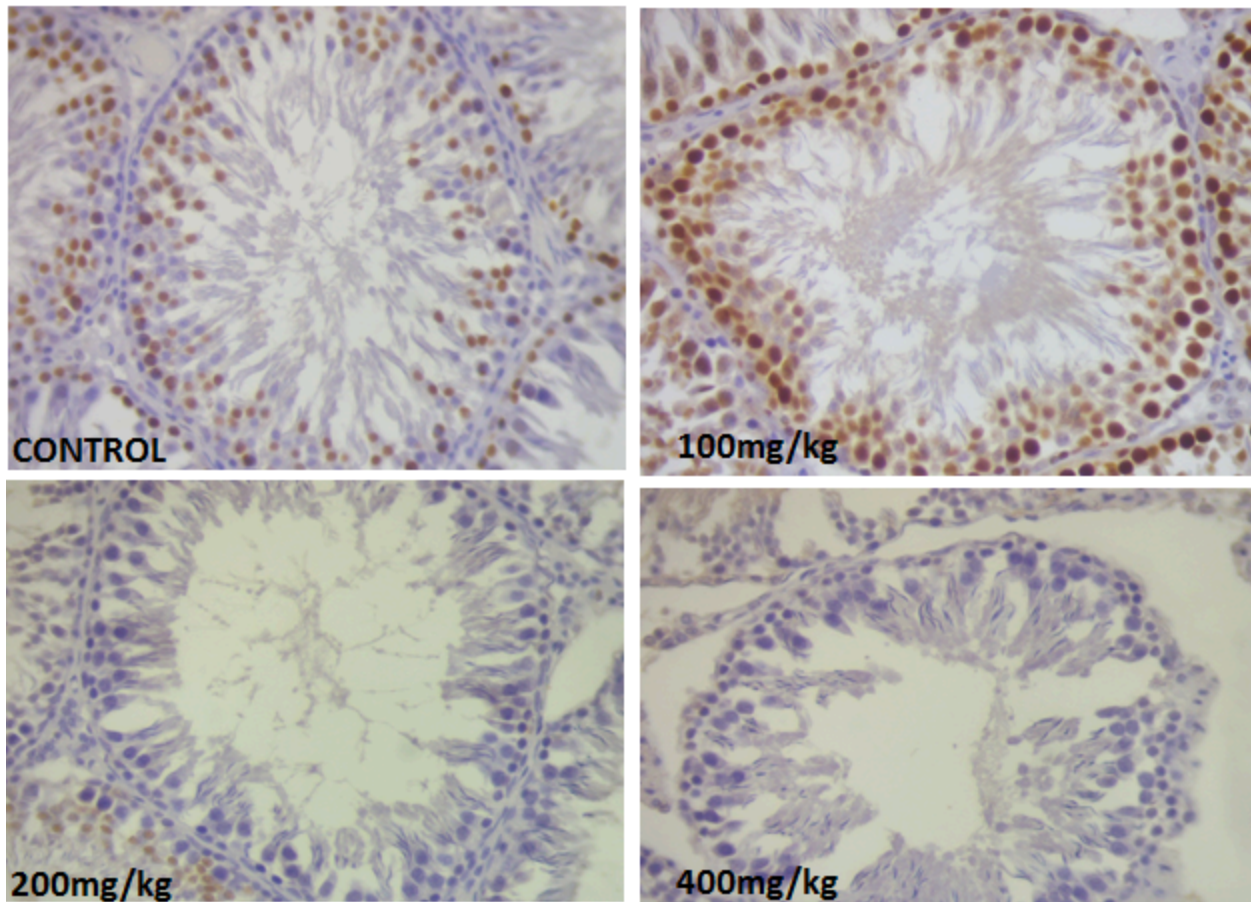
Figure 3

Effects of administering *Carpolobia alba* root methanol extract for 30 days on testicular Bcl2 immunoexpression in male rat.



**Figure 4**

Quantification of bcl-2 immunoexpression. Bars represent Mean  $\pm$  SEM (n=5) (one-way ANOVA followed by Tukey *post hoc test*). \* $p < 0.05$  relative to control.



**Figure 5**

Effects of administering *Carpolobia alba* root methanol extract for 30 days on testicular PCNA immunoexpression in male rat.

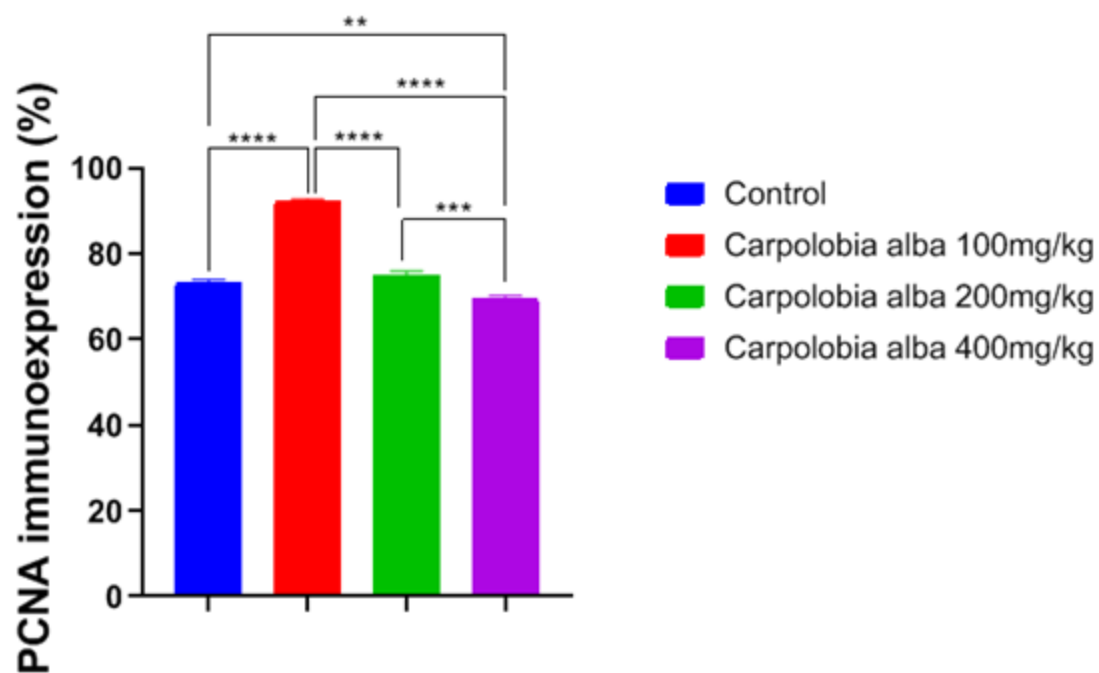


Figure 6

Quantification of bcl-2 immunoeexpression. Bars represent Mean  $\pm$  SEM (n=5) (one-way ANOVA followed by Tukey *post hoc test*). \*p< 0.05 relative to control.