



Antispermatogetic effects of seed extract of *Caesalpinia bonducella* in Swiss mice

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Abstract

Caesalpinia bonducella, a shrub profusely growing in tropical countries of the world, is known for its medicinal efficacy. However, the male antifertility effects of the plant are not at all documented. Hence, the present study is designed to investigate the male antifertility effects of the seed extract taking Swiss mice as the experimental model. The crude ethanolic seed extract of *Caesalpinia bonducella* was fed to group of sexually matured Swiss mice at a dose of 500 mg/Kg of body weight at every alternate day in such a way that every mice received 10 doses. The corresponding controls were given distilled water at the same dose and time. The experimental group of mice depicted a significant decrease ($p \leq 0.05$) in testis weight compared to controls. Sperm count in experimental groups of mice declined significantly ($p \leq 0.0001$) compared to controls. But, there was no increase in abnormal sperm count profile, testicular oxidative stress or body weight indicating that, the seed extract does not have any adverse effect in the testicular physiology. Testicular histology of the treated groups indicated intact seminiferous tubules, but, without spermatids. Flow cytometric analysis of the testis indicated cell arrest at G1 with concomitant decline in DNA synthesis at S phase and sign of tumor development. The finding of the study demonstrates that *Caesalpinia bonducella* seed extract can be used as an oral male contraceptive after specific isolation and modulation of the drug.

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Introduction

Plants are the gifts of nature which have played a significant role in maintaining human health and improving the quality of human life from time immemorial by their valuable components used commonly in food items, medicines, seasonings, beverages, cosmetics, dyes and many others. As such, herbs have attracted attention as a potential natural health care system that focuses on protecting and restoring the health (Badakhshan *et al.*, 2011).

Caesalpinia bonducella of family Caesalpiniaceae commonly known as fever nut (Nata Karanja), a prickly shrub luxuriously grown in hotter parts of India, Myanmar and Sri Lanka. Seeds consist of a thick, brittle shell with a yellowish white bitter and fatty globular kernel (Nadkarni, 1954; Handa and Kaul, 1996). More importantly, the seeds have immense medicinal value for reducing kidney problems, diabetes and high blood pressure (Chopra *et al.*, 1958; Satiyavatiet *al.*, 1976), apart from its popular use in jewellery. In addition, the seeds have a common use among rural lactating mother to protect the infants from common cold attack. Above all, it is also reported to have multiple therapeutic properties like adaptogenic and antidiabetic (Kannur *et al.*, 2006a; Kannur *et al.*, 2006b), antitumor (Gupta *et al.*, 2004), antifilarial (Gaur *et al.*, 2008), immunomodulatory (Shukla *et al.*, 2009a), antioxidant (Shukla *et al.*, 2009b), anti-inflammatory, antipyretic, analgesic (Shukla *et al.*, 2010), hypolipidemic (Sharma *et al.*, 1997; Chakrabartiet *al.*, 2003), muscle contractile activity (Datteet *al.*, 2004), antimicrobial (Arif *et al.*, 2009) and diuretic agent (Khedkaret *al.*, 2011). *C. bonducella* seeds are used by the tribal people in India for controlling blood sugar and asthma and chronic fever in Tanzania (Nadkarni and Nadkarni, 1976; Moshi and Nagpa, 2000; Chakrabarti *et al.*, 2003).

However, recent demonstration of *Caesalpinia bonducella* seed extract for antifertility efficacy in females have apparently been proved successful for the control of fertility over hormonal means.

Alternately, some literatures demonstrate that the seed extract of *Caesalpinia bonducella* could be used as an anti-fertility agent in females (Lilaram, 2012). Similarly, preliminary studies of the root bark extract indicates its anti-implantation effect in rats (Sukhdev, 2011).

However, use of *C. bonducella* products as male antifertility agent has not yet been tested. Hence, the present project has been undertaken to test the male antifertility efficacy of *C. bonducella* seed extract taking Swiss mice as the experimental model.

Materials and methods

Preparation of the seed extract

Seeds of *Caesalpinia bonducella* were collected around Berhampur University (Odisha, India). The seed extract was prepared following the method (Shukla, 2009a). Seeds were air-dried at room temperature in the laboratory, for seven days after which, the semi solid mass was mechanically grinded to a powder form.

The powder was subjected to solvent extraction method using a Soxhelt apparatus. For the extraction procedure ethanol was taken as solvent. After successful cycles of soxheltation, the crude samples were separated and left for normal evaporation of ethanol. The crude extract so collected was kept at 4°C for further use.

Animal model

Healthy and sexually matured male Swiss albino mice (15-25gms body weight) were purchased from M/s Ghosh Enterprises, Kolkata, India and were reared in the animal house which is maintained at 25 °C ± 2°C temperature and with a constant 12 hour light and dark cycle. The mice were kept in healthy condition with a daily feed of balanced diet and water ad libitum.

Treatment protocol

Mice were taken for present study and were divided into control and experimental groups (n- 6) of mice. The control group of mice was orally supplemented

with distilled water and the experimental group of mice was orally supplemented with *C. bonducella* seed extract at a dose of 500mg/kg body weight, on every alternate day for ten days. Mice of both the groups were sacrificed on the next day after the completion of treatment.

Semen analysis

Sperm count and sperm head abnormality studies were done following the procedure (Wyrobek and Bruce, 1975). Sperm were squeezed out from the vas deferens in phosphate buffer saline (PBS) at room temperature, aspirated gently by pasture pipette and left for five minutes. Samples were centrifuged for ten minutes at 1000 rpm and the supernatant was discarded. A little amount of PBS added and aspirated gently to prepare a thick homogeneous suspension of sperm in PBS. A small drop of sperm suspension was taken on dry and grease free slides, smeared gently and left overnight for natural drying. Next day, slides were dipped in double distilled water for 2 to 3 min. and stained with 10% Giemsa stain (pH=6.8) for 1 -2 hours.

The slides were washed thoroughly in tap water and observed under microscope for abnormality studies. About 1000 sperm from each specimen were scanned. Morphologically, abnormal sperm were recorded as described (Wyrobek and Bruce, 1975). Sperm count was done by a hemocytometer. Enumeration of sperm was done in RBC counting chamber and the calculation was done as per RBC enumeration formula.

Histology of testis

The testes were recovered from the mice, cleaned off accessory tissues and small pieces of testes were fixed in ice-cold formal-calcium solution for a period of 24 to 48 hours. Then formal calcium fixed tissues were washed thoroughly in tap water and dehydrated in different grades of ethanol and paraffin blocks were prepared. Microthin sections (5 μ) were cut using a microtome machine. Testes section were stained with haematoxylin-eosin stain (Pearse, 1975) and observed under the microscope to study the

histological details.

Testicular oxidative stress

Oxidative stress of the testes was measured following the method (Sestini *et al.*, 1991) with slight modification as described (Jena *et al.*, 1995).

Cell Cycle Kinetics by Flow cytometry

For the study of cell cycle kinetics, flow-cytometry procedure was followed using paraffin-embedded testes tissue, as described (Hedley *et al.*, 1983) and subsequently modified (Blanco *et al.*, 2013). 50 μ m paraffin sections from the tissue block were dewaxed in xylene followed by rehydration in descending granules of ethanol and finally washed in distilled water. The tissues were treated with sodium citrate antigen retrieval solution pH 6 for 2h at 80°C and then with 0.5% pepsin solution (pH 1.0) at 37°C for 10 min.

The solution was centrifuged (100 rcf) and the supernatant was removed. Single-cell suspension was filtered through 40 μ m nylon mesh. Then the cell suspension was stained with propidium iodide (Sigma P4170) at a final concentration of 40 μ m/mL. A number of 10,000 nuclei were acquired for each sample, at a low flow rate, using a BD FACSCalibur flow cytometer (BD Biosciences, USA) equipped with dual-laser. The data obtained was analysed using the FCS Express software version 5.0 (De Novo Software).

Histogram was considered unsuitable for interpretation if its variation coefficient (CV), exceeded 10% than the normal cell population. A sample is considered DNA diploid if, on the histogram, there is a single peak in the G₀/G₁ phase. DNA aneuploidy was defined if, there was at least one separate second G₀/G₁ peak.

Statistical analysis

Data degenerated out of seed extract-treated mice groups were compared with the control mice. The significance of the data was verified by students' 't' test as described (Garret, 1956). 'P' the value of

significance was found from a 't' table (Abramoff and Thomson, 1966; Bishop, 1967). 'P' value at and below 0.05 level were considered significant.

Results

Behavioral, body and organ weight profile

There were no treatment related changes in the behavioral profile of the mice groups at all the tested

dose levels. All animals in seed extract-treated groups appeared healthy, alert and were responding to pain and touch. Vocalization, restlessness and irritability in animals were also not observed. The animals responded to loud noise, indicating the CNS excitation. The treated mice groups depicted a significant increase in body weight ($p \leq 0.05$) compare to the untreated controls (Fig-1).

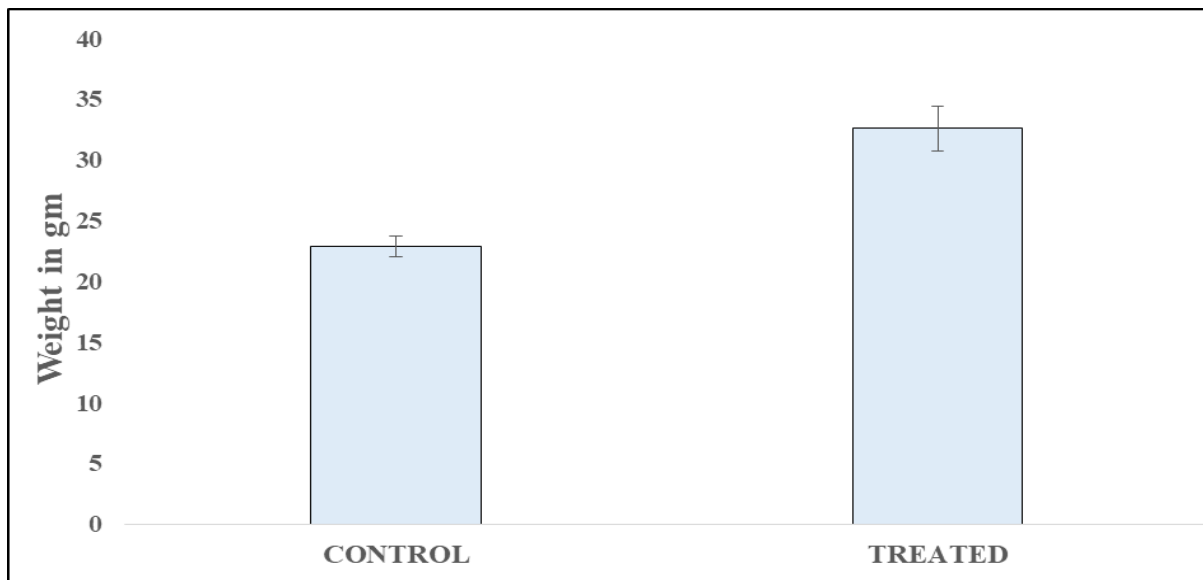


Fig. 1. Showing the effect of *C. bonducella* seed extract on Body weight (gm) of Swiss mice. ($P = NS$).

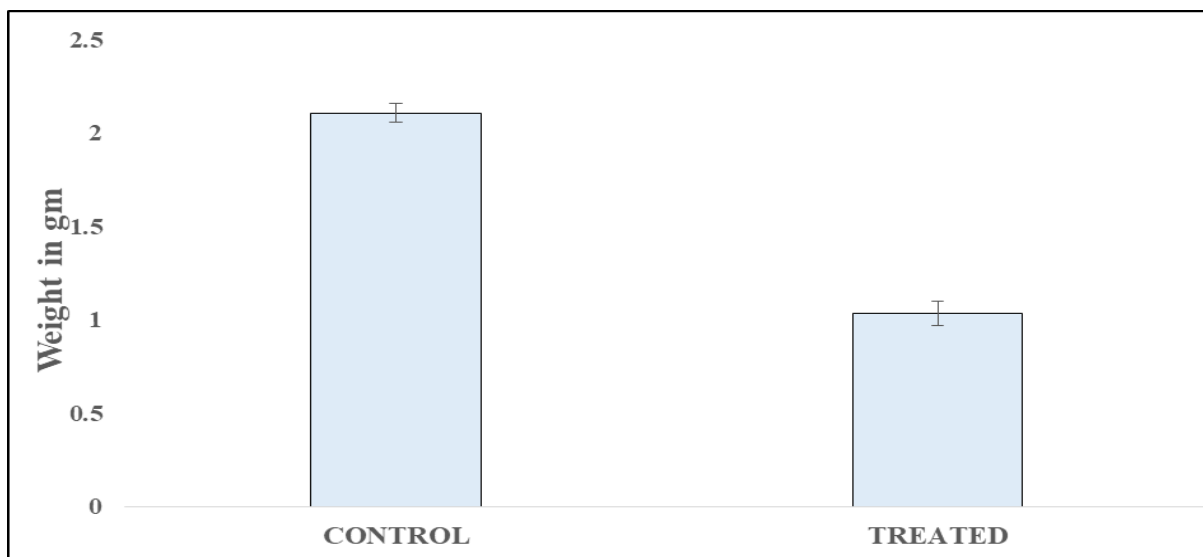


Fig. 2. Showing the effect of *C. bonducella* seed extract on Testis weight (gm) of Swiss mice. ($P \leq 0.05$).

However, it was observed that the mice groups indicated significant decrease ($p \leq 0.05$) in testes weight after exposure to *Caesalpinia bonducella* seed extracts when compared with the untreated controls (Fig-2).

Sperm quality and quantity

Sperm count in seed extract-fed mice was declined significantly ($p \leq 0.0001$) relative to untreated controls (Fig-3).

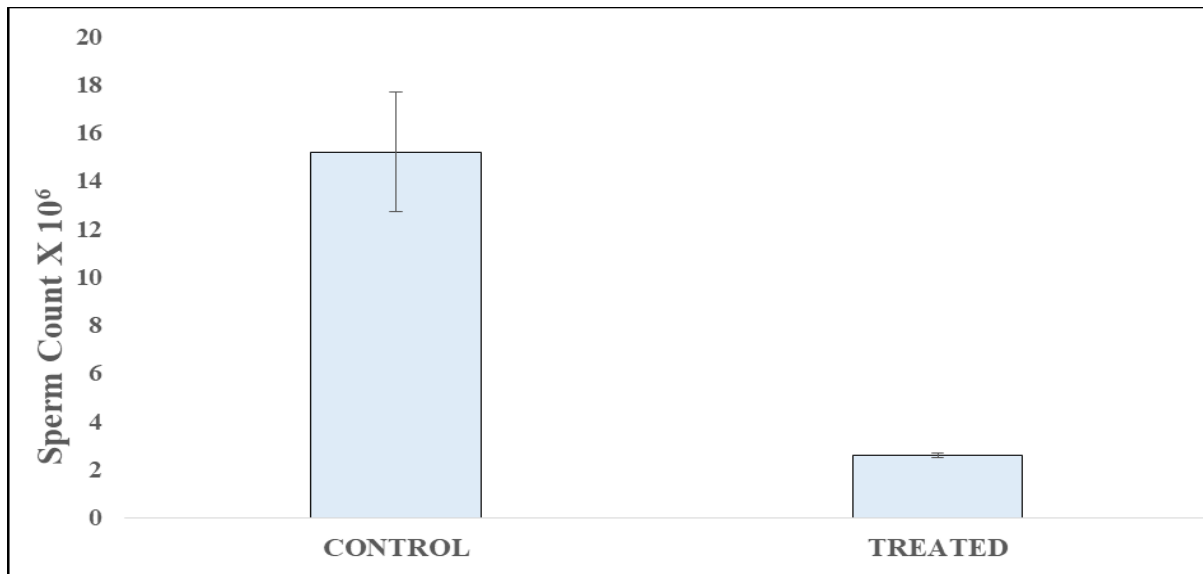


Fig. 3. Showing the effect of *C. bonducella* seed extract on sperm count x (10^6) of Swiss mice. ($P \leq 0.0001$).

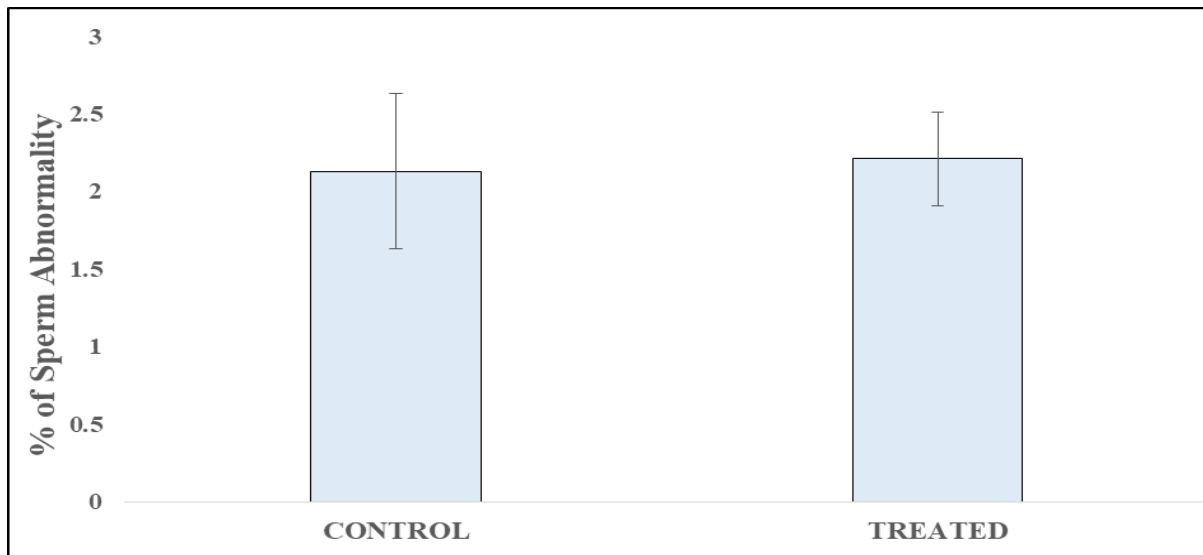


Fig. 4. Showing the effect of *C. bonducella* seed extract on sperm abnormality (%) of Swiss mice ($P \leq 0.05$).

It was also found that in treated mice groups. There was no significant increase in the no. of morphologically abnormal sperm population compared to the untreated controls (Fig-4).

Testis histological observation

In *C. bonducella* seed extract-treated mice groups, the seminiferous tubules contains the spermatocytes but without spermatozoa and some of the seminiferous tubules filled with fluid. No attachment of spermatids on sertoli cells (Fig-5). Some of the seminiferous tubules are degenerated with the loss of peritubular cells and spermatogonial cells (Fig-6).

The damaged seminiferous tubules mostly contain apoptotic cells with marginal nuclei or necrotic spermatocytes (Fig-7).

Cell cycle kinetics study

Flow cytometry studies of the testes sections indicate the distribution of cells in different phases of cell cycle. From the study it is clearly evident that in the *C. bonducella* exposed mice groups, gametogonial cells have been arrested at G1 and few committed cells have been passed to S phase (Fig: 9). Similarly, DNA content analysis of both control and experimental the sections indicate the heavy amount

of DNA in G1 and very less amount of DNA at G2 phase, which further supports the arrest of G1 cells (Fig. 10). It also clearly observe from the cell proliferation statistics that most of the cells are get arrested at 1st generation of the cell cycle i.e. at G1 phase in the *C. bonducella* exposed mice groups (Fig. 11 and 12).

Discussion

The results of the present study demonstrate the

drastic decline in the sperm count of bonducella seed-extract-fed mice groups with respect to control groups. This is probably due to necrosis and/or of apoptotic phenomena. However, testicular oxidative stress does not show any increased generation of singlet oxygen species in the experimental group of mice compared to controls. Hence, oxygen-dependent mechanisms could possibly be precluded for sperm count decline.

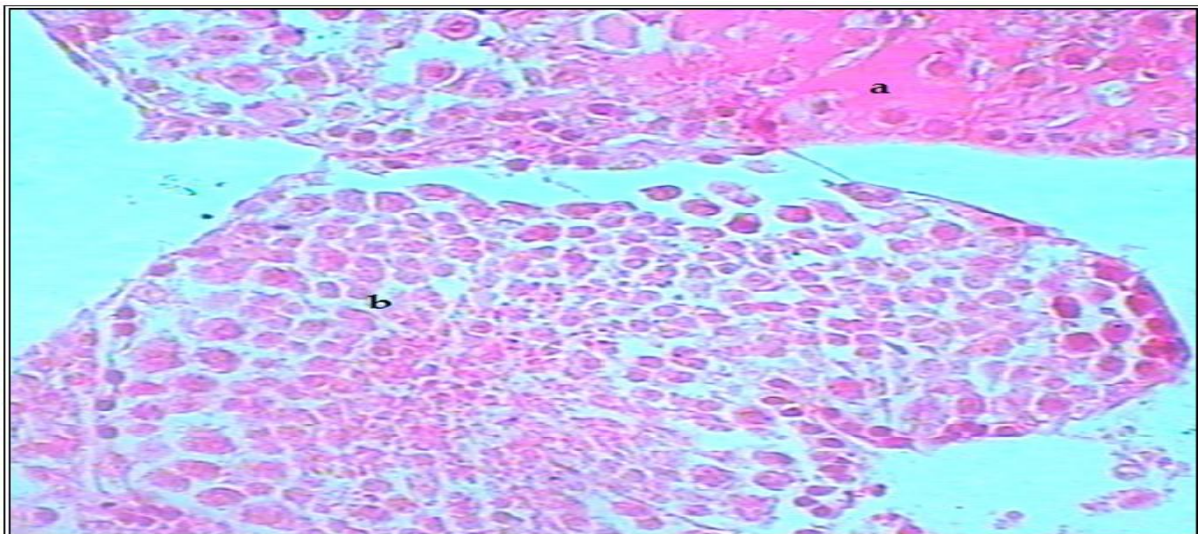


Fig. 5. Photomicroscopic pictures of testis showing fluid filled seminiferous tubules (mark 'a') and sertoli cells with no attachment of spermatids (mark 'b').

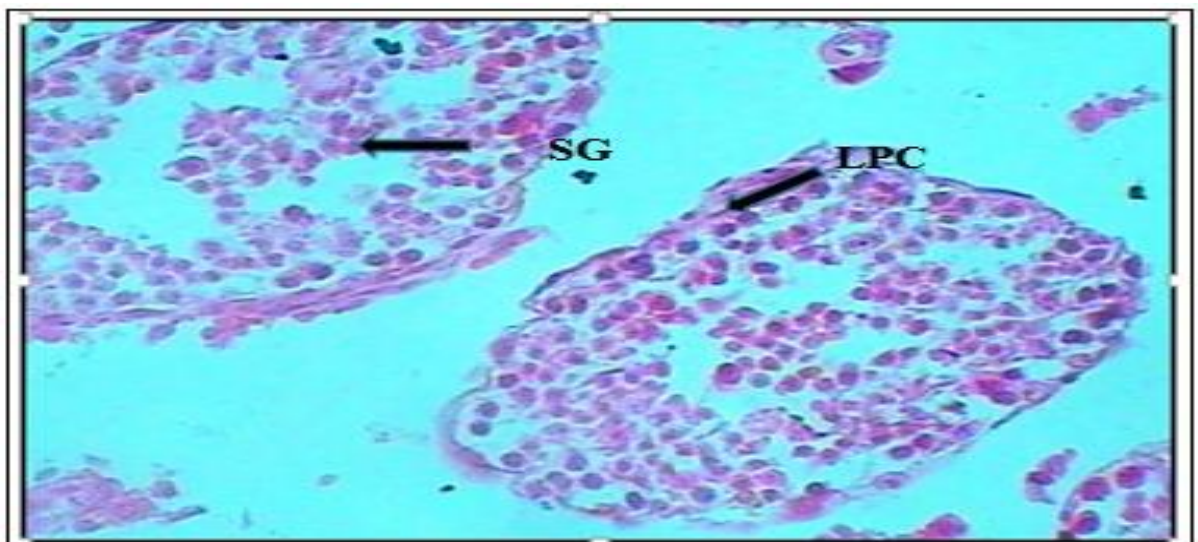


Fig. 6. Photomicroscopic pictures of testis showing the loss of peritubular cells (LPC) and spermatogonium (SG).

The present authors are not in conformity with the earlier workers (Preeja and Suresh, 2011) with regards to generation of oxyradicals by *C. bonducella* extract.

Furthermore, significantly lower sperm count in experimental mice groups is possibly linked with the arrest of cell cycle resulting in the non-transformation of spermatids into matured sperm.

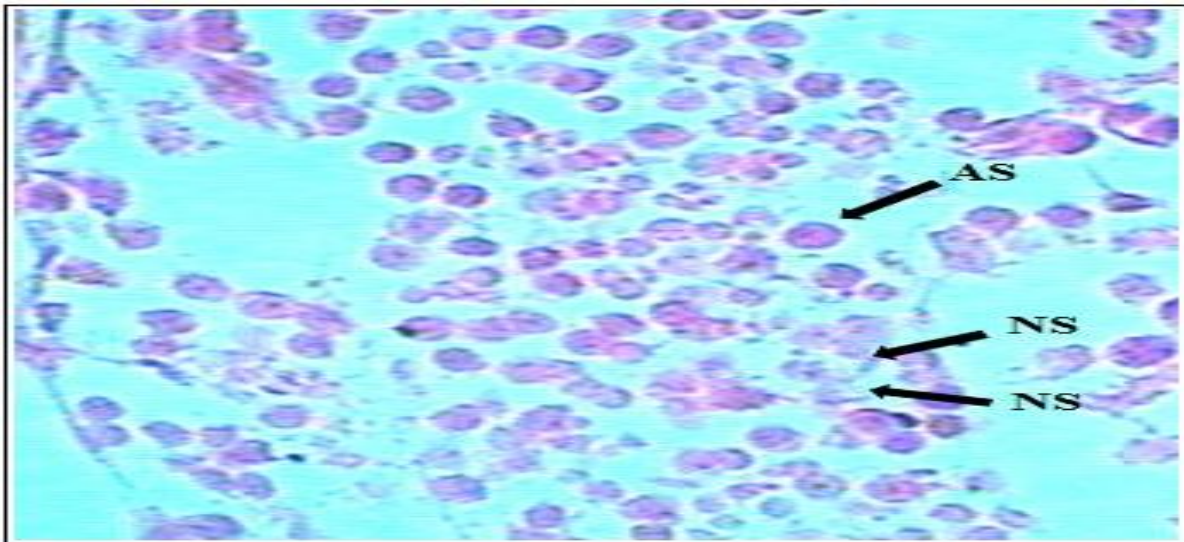


Fig. 7. Photomicroscopic pictures of testis seminiferous tubules showing apoptotic (AS) and necrotic spermatocytes (NS).

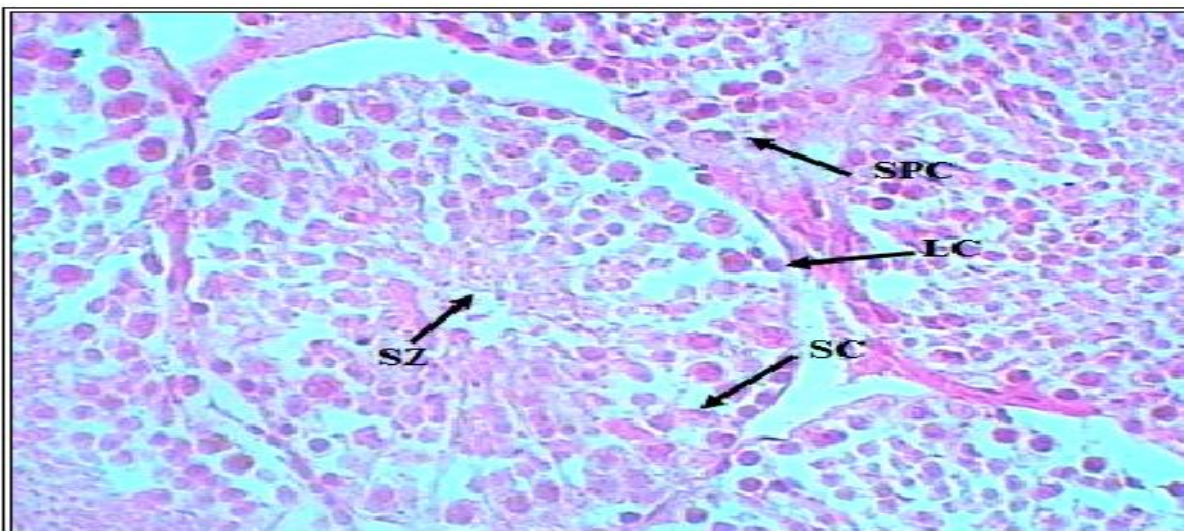


Fig. 8. Histoarchitecture of control testis showing seminiferous tubules (SZ) with spermatocytes (SPC), sertoli cells (SC), and cells of leydig (LC).

This is well indicated in the eosin stained testis sections, where the tubules are laden with developing gametogonial cells at different stages of development, but without forming any matured sperm.

This is ascertained as by flow-cytometric data which rightly demonstrates the arrest of cell cycle at G1. When the data of experimental testes is compared with the controls, it firmly demonstrates the accumulation of cell debris which are formed due to necrosis and/or apoptotic cells.

In the present context, such accumulation is possibly due to G1 arrested cells or else, the spermatocytes which fail to attach to sertoli cells for further transformation and differentiation into spermatids are getting degenerated and accumulated as cell debris. Furthermore, flow-cytometric data also states the occurrence of G1 peak before 256 nm indicating cell arrest at G1.

This indicates that only commitant cells could be able to pass into S phase. Since majority of the cells are arrested as a result of the potential activity of seed extract, the measurement of DNA in S phase is drastically depleted compared to controls.

This is also reflected in G2 and M. Another interesting finding of the present study is that *bonducella* seed extract is not at all a tumour causing agent, which is confirmed from the flow chart. Since, majority of the cells are arrested in G1, it also speaks us that

bonducella seed extract might be a protein inhibitor, probably preventing cell cycle proteins like cyclins or CDKs that propel the cells from G1 to S. This contention, however needs further research.

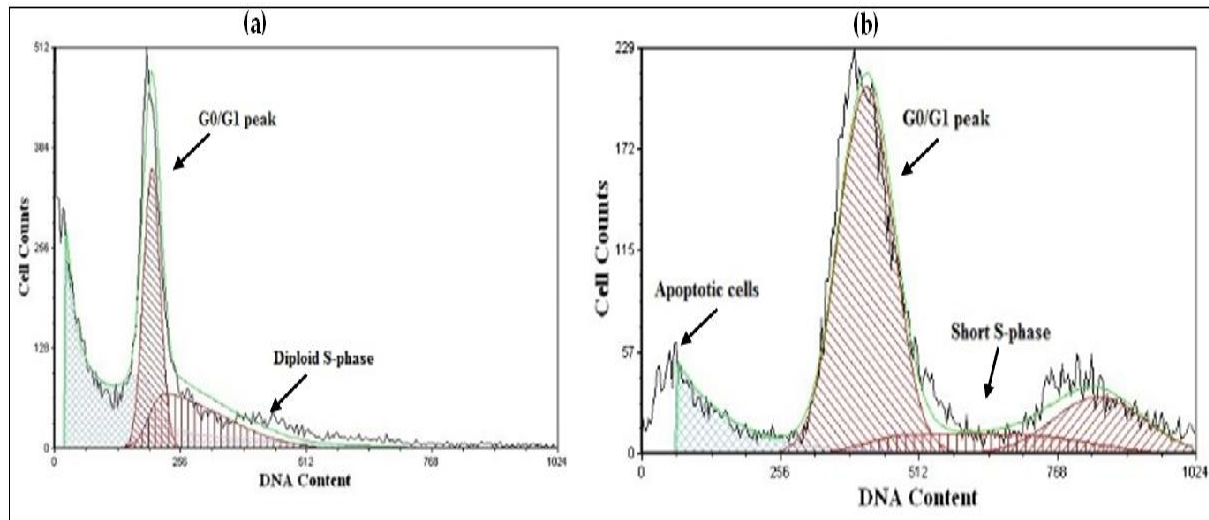


Fig. 9. DNA content analysis of testes sample measured by the flow cytometric profile and analyzed by FCS Express 5.0 software. Cells prepared by the Blanco method²⁷ and stained with propidium iodide. (a) DNA histogram of control group. Note a single G0/G1 peak and diploid S-phase. (b) DNA histogram of *C. bonducella* treated group showing unusual peak for undivided cells (mostly apoptotic cells) before G0/G1 peak and a very short S-phase.

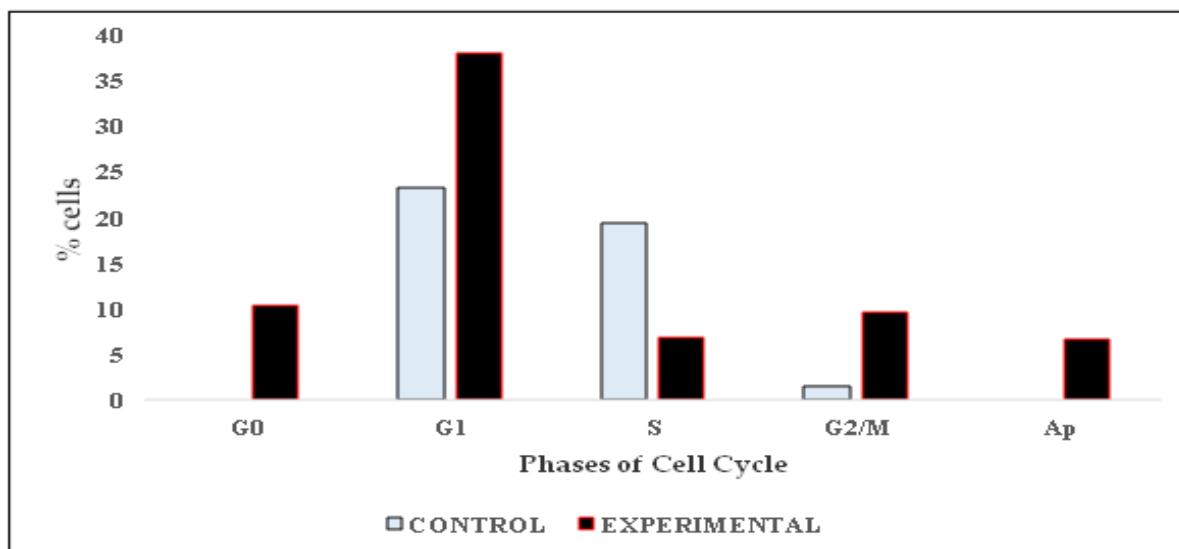


Fig. 10. Analysis of % cells of testes sample measured by the flow cytometric profile and analyzed by FCS Express 5.0 software. Cells prepared by the Blanco method and stained with propidium iodide.

Here in the present study, *C. bonducella* seed extract fed mice groups exhibit significantly reduction in sperm count profile compared to control group of mice. It is generally believed that defective sperm

function is the most prevalent cause of male infertility, and is difficult to treat. The ROS production due to oxidative stress is produced by a variety of semen components, including immotile or

morphologically abnormal spermatozoa, leukocytes and morphologically normal but functionally abnormal spermatozoa. Earlier studies it has been reported that, oxidative stress is associated with a reduction in sperm motility and viability (Baumber *et al.*, 2000). In addition several reports have indicated that, *C. bonducella* seeds have anti-oxidative potential by scavenging oxy-radicals and enhancing the concentration of several antioxidant enzymes (Kumar *et al.*, 2005; Shukla *et al.*, 2009b; Mandal *et al.*, 2009; Singh and Raghav, 2012).

Hence, the present authors consider that sperm count decrease or reduction in sperm mobility or viability in

the present situation might not have been through enhanced oxidative stress. On the other hand, the involvement of hormones like FSH and LH in shaping spermatogenesis in *C. bonducella* seed extract fed mice groups may possibly be overruled because of the healthy development of spermatocytes from spermatogonia in the seminiferous tubules and the occurrence of healthy leydig cell mass. Conversely, reports are hardly available to describe the potential effects of bonducella seed on hormonal alteration in spermatogenic pathways. Hence, the present authors are skeptic with regards to the involvement of hormonal mechanisms for spermatogenesis disruption leading to sperm count decrease.

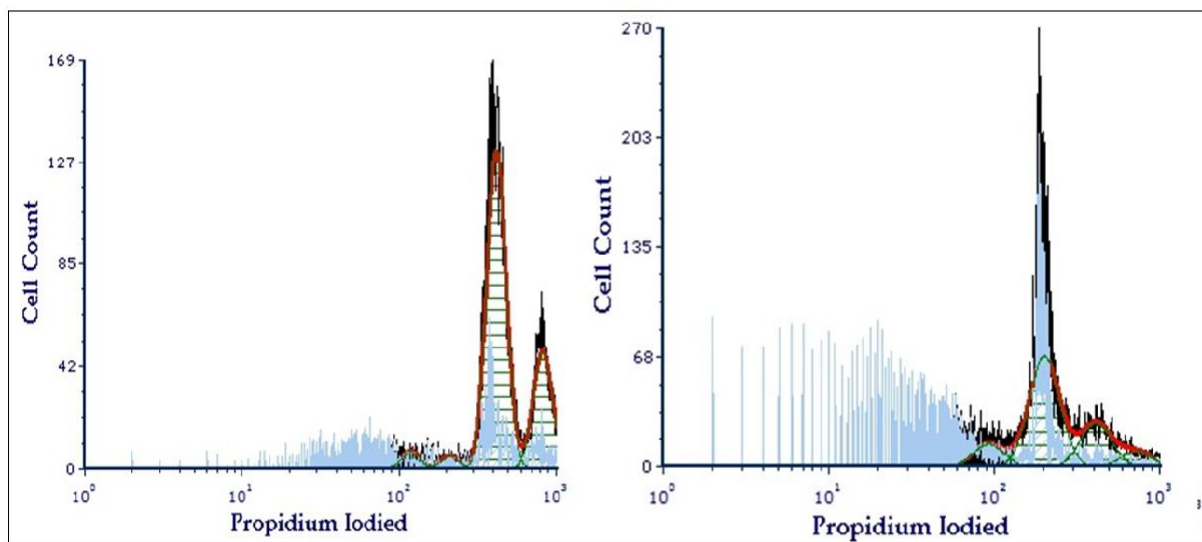


Fig. 11. Showing Cell Proliferation during the events of Cell Cycle measured by Flow cytometry. Cells are prepared by Blanco method²⁷ and stained with propidium iodide. (a) Control and (b) *C. bonducella* treated.

Of note, *C. bonducella* seed extract treated mice groups did not reflect any sign of tumor development. Had it been there, multiple and repeated peaks in the flow chart must have been occurred.

Essentially, this excludes the drastic possibility of tumorigenic potentialities of *C. bonducella*. Moreover, from the study we can probably suggest that *C. bonducella* might be acting as a potential protein inhibitor which probably down regulates the synthesis of late G1 cdks and cyclins which are crucial in propelling the G1 cells to S phase. Secondly, we may conclude that *C. bonducella* seed extract might be working as a tumour suppressor by upregulating

the p53 protein synthesis via p21 protein expression by which G1 cell arrest has become possible.

This finding is in agreement with the previous studies indicating anti-tumour activity of the seed (Gupta *et al.*, 2004; Yadav *et al.*, 2009).

The findings of the present study, focuses the antifertility efficacy of *C. bonducella* seed extract by cell arrest at G1 phase.

The active compound may be identified, isolated in pure form and can be utilized for the preparation of oral contraceptives for males.

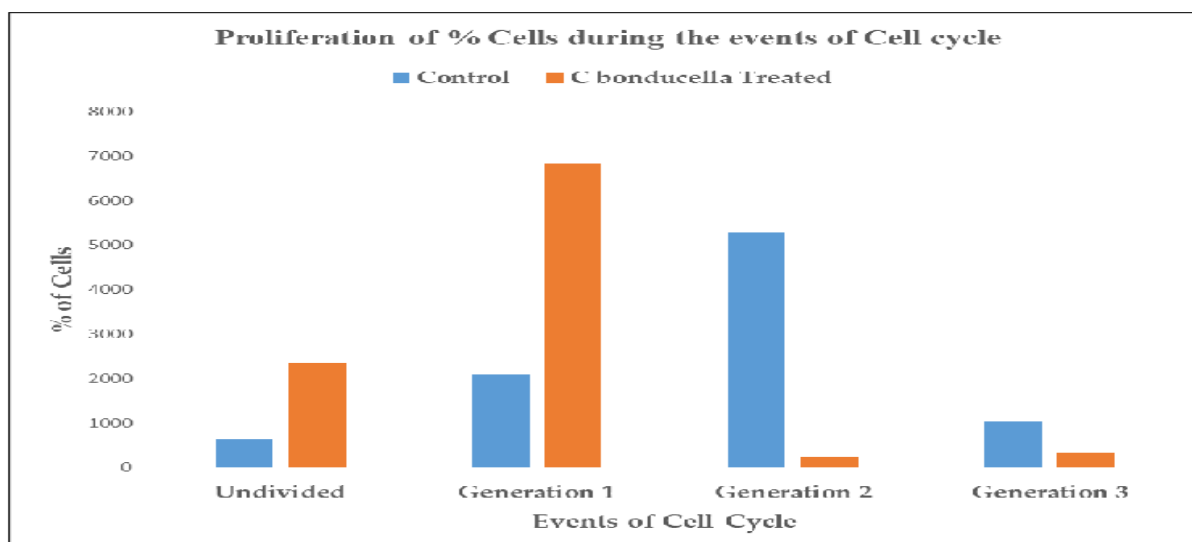


Fig. 12. Showing statistics of % Cells proliferated during the events of cell cycle. It is clearly observe that the cells are get arrested in the generation 1 of the *C bonducella* treated category in comparison to the control.

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