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Full Length Research Paper

Antifertility effects of ethanolic seed extract of *Abrus precatorius* L. on sperm production and DNA integrity in adult male mice

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Abrus prectaorius **L. is one of the folk medicinal plants widely used as an antifertility agent in various places of Pakistan. The aim of the present study was to investigate the antifertility activity of *A. prectaorius* seed extract intraperitoneally administered on sperm production and DNA integrity of spermatozoa in adult male albino mice of BALB/c strain. The daily sperm production was measured by counting testicular spermtids in Horwell chamber while DNA damage in epididymal spermatozoa was determined by comet assay. The intraperitoneal administration of 20 and 60 mg/kg of ethanolic seed extract of *A. precatorius* caused a highly significant ($p \leq 0.001$) decrease in daily sperm production. The reversibility in sperm production was observed in all the treated animals after 20 days of withdrawal of treatment. Similarly, a highly significant increase ($p \leq 0.001$) in DNA damage was observed in all the treated animals and no significant reversibility in DNA damage was observed during treatment period. This study suggests the role of seed extract of *A. precatorius* as an antifertility agent or contraceptive with a risk of DNA damage in spermatozoa and may lead to teratogenic effects.**

Key words: Antifertility, *Abrus precatorius* L., sperm production, DNA damage.

INTRODUCTION

Plant species

Abrus precatorius L. (Paplionaceae) is located in Khauta Punjab, Pakistan and authenticated by QAU, Herbarium of Pakistan. A voucher specimen has been preserved in the laboratory for future reference.

Therapeutic uses

A. precatorius L. is one of the best antifertility regulating herbs being widely used in south Asian countries (Ross, 2005).The tribal communities of Potohar region Pakistan, use seeds of *A. precatorius* in order to control child birth, as purgative, emetic, tonic, aphrodisiac, anti-ophthalmic, antiphlogistic and aphrodisiac (Ahmad et al., 2008).

Chemical constituents

Glucoside, abrusic acid, haemagglutinin, poisonous proteins, a fat splitting enzyme and abrin (Nadkarni, 1954). Cycloartane glycoside, designated abrusoside E, 3β-O-[β-D -glucuronopyranosyl-(1 → 2)-β-D -glucopyranosyl] (20S, 22S)-3β, 22-dihydroxy-9, 19-cyclolanost-24-en-26, 29-dioic acid δ-lactone (Kennelly et al., 1996).

Antifertility activity

Methanolic seed extract shows contraceptive effect in adult male mice (Bhatt et al., 2007). Similarly its ant motility effect has been demonstrated on washed human spermatozoa (Ratnasoorya et al., 1991). *In vitro* studies

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of isolated abrin protein from *A. precatorius* exhibit oxidative activity leading to DNA fragmentation in somatic cells (Shih et al., 2001; Qu and Qing, 2004; Narayanan et al., 2004; Bhaskar et al., 2008 and Bhutia et al., 2008) indicating a possibility of DNA damage induction by *A. precatorius* in spermatozoa along with its antifertility activity. The present study is designed to evaluate the antifertility activity of ethanolic seed extract of A. precatorius regarding daily sperm production and DNA damage in spermatozoa in a mouse model.

MATERIALS AND METHODS

Plant material and extraction

Whole plant specimens with dried seeds were collected during the field trips and also procured from herbal shops. The plant material was authenticated by comparing with the Herbarium specimens, Quaid-i-Azam University Islamabad, Pakistan. An ethanolic seed extract was prepared using the cold extraction method (Adome et al., 2003). The seeds were coarsely powdered in electrical grinder (Disk Mill Model FFC-15). 500 gram of seed powder was soaked in 99.9% ethanol in a conical flask for 1 week and the contents were filtered. This filtrate was evaporated under reduced pressure in rotary vacuum evaporator and then dried at room temperature. The dried mass was scraped at the bottom of the flask and stored at 4°C. The yield of extract was 0.6% of the raw material. Two different doses of 20 and 60 mg/kg/BW were prepared from the stock solution obtained by dissolving 3 g of the extract in 1000 ml of physiological saline.

Animals

Adult male albino BALB/c mice of 30 - 35 days age (n = 48) were used. They were housed at primate facility in steel cages with standard laboratory diet and water ad libitum for two weeks. The study was conducted after the permission of Institutional Animal Ethical Committee.

Treatment

The male mice were divided into four groups of twelve animals each; Group I, control; mice in group II and III received 20 and 60 mg/kg doses of extract intraperitoneally for 20 days, respectively. After 20 days of treatment, half of the animals from each group were sacrificed. The remaining animals from each group were sacrificed after 20 days of withdrawal of treatment. Testes and epididymis were removed from each animal and processed for analysis of daily sperm production and DNA damage, respectively.

Daily sperm production (DSP) determination

Testicular sperm production was determined by the procedure of (Cooke et al., 1991). Testis was weighed after removal and then their tunica albugina was removed, which was also weighed. Testis was homogenized for 20 min in 15 ml homogenizing solution (0.05% Triton X-100, 0.01% sodium azide) using an electrical homogenizer. In order to count spermatids Horwell chamber was used where each 0.2 ml sample of homogenate was diluted with 0.8 ml of saline solution containing 1% trypan blue. Sample aliquots of 10 µl were placed on the Horwell chamber and average number of spermatids per sample aliquot was counted under the 400× magnification of microscope. This number was multiplied by dilution factor and original volume of homogenate to get total testicular spermatid count which was divided by weight of decapsulated testis to get number of spermatids per gram of testis. Developing spermatids spend approximately 4.84 days in steps 14 - 16 during spermatogenesis in mice. Thus the values for the numbers of spermatids per testis and per gram testis were divided by 4.84 to obtain daily sperm production (DSP) and the efficiency of sperm production, respectively (Thayer et al., 2001; Joyce et al., 1993).

DNA Damage by SCGE or comet assay

The DNA status of individual spermatozoa was determined using a modified alkaline single cell gel electrophoresis (SCGE) / comet assay (Bhaskar et al., 2008). The epididymal spermatozoa were collected by removing the excessive fats and then minced with forceps in phosphate buffer saline (PBS) solution at 37°C. The spermatozoa were diluted in PBS to get 10,000 sperms in 10 µl of cell suspension. Clear window frosted microscopic slides were coated with 1% normal melting agarose. Aliquots of 10 µl of cell suspension were mixed with 70 µl of 0.5% agarose at 37°C. The slides were laid on icepack for 2 - 3 min, followed by another coating of 70 µl of agarose and placed on icepack until the agarose layer hardens. The slides were placed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, 40 mM Dithiothrietol, pH 10) for overnight at 4°C. Next day slides were dipped in lysing solution containing protienase K for 2 h at 37°C in dark.

After lysis, the slides were immersed in the fresh electrophoresis buffer (10 mM Tris, 0.08M Boric Acid, 0.5M EDTA, pH 8.2) and incubated for 30 min. at 4°C for DNA unwinding and alkali labile site expression and electrophoresed for 20 min. at 25 V and 300 mA in electrophoresis tank. After electrophoresis the slides were drained, dehydrated by immersing the slides in absolute ethanol for 3 - 5 min, dried at room temperature and stored in a slide box. The slides were then stained with acridine orange in dark room and left for 5 min. then washed with distilled water and air dried. The slides were observed under fluorescent microscope (Nikon) and photographed by cannon digital camera. Comets were scored in photographs by using TRITEK software the comet parameters considered in this study were number of comets, comet length (µm), comet height (µm), comet area (µm²), tail length (µm), %DNA in tail, tail moment and olive moment.

Statistical analysis

All values expressed as mean ± S.E.M. were analyzed and compared by Turkey's post hoc test. Comparison between the treated and withdrawal groups was determined by using student's t test.

RESULTS

The present work provides compelling evidence for the antifertility activity along with genotoxic effects in adult male albino mice. To date the antifertility effects of *A. precatorius* have been restricted to sperm concentration, morphology, motility and viability (Rao, 1987; Ratnasooriya et al., 1991; Bhatt et al., 2007) and fertilizability (Sinha and Mathurm, 1990). In some advanced studies, steroidal fraction has been isolated from the seeds of A. precatorius and found to exhibit antifertility activity (Sinha and Mathurm, 1990). As far as evaluation of DNA damage by *A. precatorius* is concerned, it has

Table 1. Mean testicular sperm count and daily sperm production in control and A. precatorius treated adult male albino mice.

Groups	Control	20 mg/kg	60 mg/kg	Withdrawal	Treated	Withdrawal
	Treated	Withdrawal	Treated			
Testicular sperm count (10^7)	6.42 ± 0.14	5.76 ± 0.55	1.87 ± 0.10^{a**}	4.46 ± 0.07	1.53 ± 0.23^{a**}	5.22 ± 0.22
Daily sperm production (10^7)	1.33 ± 0.03	1.19 ± 0.11	0.39 ± 0.02^{a**}	0.92 ± 0.01	0.32 ± 0.05^{a**}	1.08 ± 0.04
No. of sperms per gram testis(10^8)	8.45 ± 0.44	8.73 ± 0.59	2.52 ± 0.07^{a**}	7.23 ± 1.21	3.05 ± 0.53^{a**}	5.78 ± 0.69
Efficiency of sperm production(10^7)	17.39 ± 0.96	18.00 ±0.12	5.21 ± 0.15^{a**}	14.90 ±0.25	6.24 ± 1.03^{a**}	11.90 ± 0.14

Values (Mean ± SEM), a = control vs. treatment groups (20 and 60 mg/kg), $^{**}p \leq 0.001$.

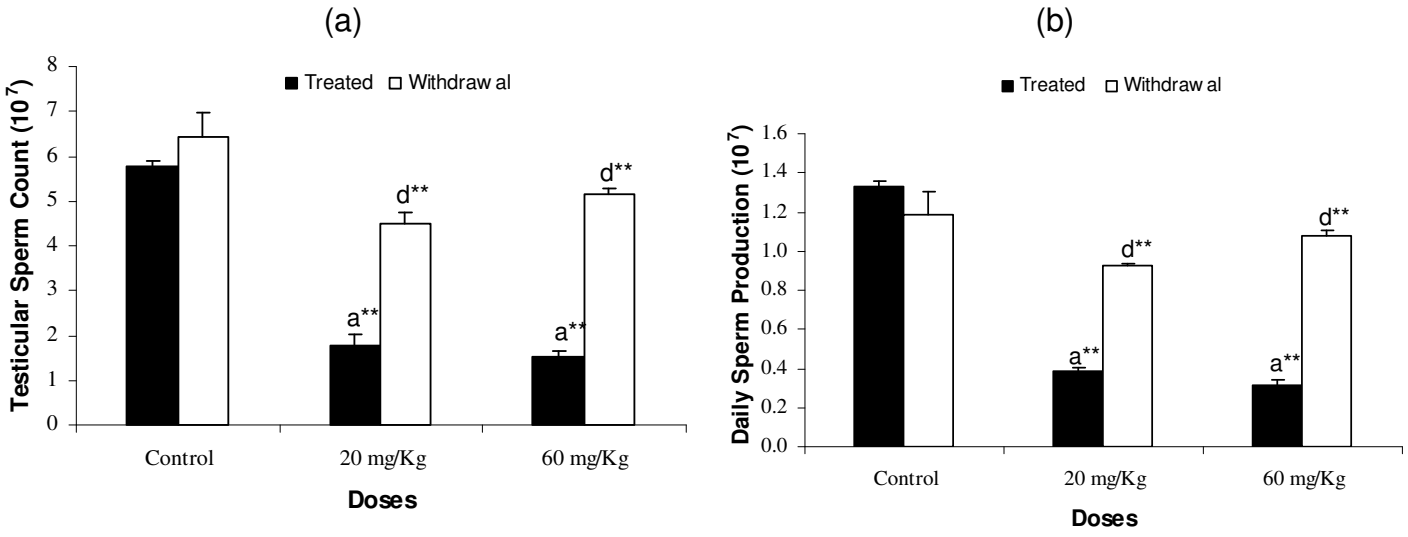


Figure 1. (a) Mean testicular sperm count and (b) Mean daily sperm production in control, A. precatorius treated male albino mice and after 20 days of withdrawal of treatment.
Values expressed as Mean ± SEM, a = control vs. treatment groups (20 and 60 mg/kg), d = Treated vs. Withdrawal, **P ≤ 0.001

been demonstrated that isolated constituents from the seeds of *A. precatorius* that is, abrin and agglutinin induce apoptosis by causing DNA fragmentation in vitro (Qu and Qing, 2004; Bhaskar et al., 2008) but induction of DNA damage in spermatozoa by administration of seed extract is still undetermined. A striking finding of the present study is that *A. precatorius* seed extract has genotoxic effects on DNA of spermatozoa in adult male mice.

Daily sperm production (DSP)

Ethanoic seed extract of A. precatorius induced a highly significant ($p \leq 0.001$) decrease in mean testicular sperm count and daily sperm production in all the treatment groups compared to control (Table 1, Figures 1a and 1b). Similarly a significant decrease ($p < 0.05$) was observed in mean sperm count per gram of testis and efficiency of sperm production in both treated groups compared to control (Table 1, Figures 2a and 2b). The levels re-covered to almost normal after 20 days of withdrawal of treatment in both treated groups.

DNA damage assessment

The number of comets for treated animals is shown in Table 2. Comparison of treated groups with control group showed a significant increase ($p < 0.05$) and a highly significant increase ($p \leq 0.001$) in comet numbers in 20 mg/kg and 60 mg/kg treated groups, respectively, compared to control. A highly significant ($p \leq 0.001$) dose dependent increase in comet numbers was observed within the treatment groups (Figure 3). A highly significant increase ($p \leq 0.001$) in comet length, height and area in both the treated groups was observed compared to control as shown in Table 2. Tail length of 20 mg/kg showed a significant increase ($p < 0.05$) as compared to control while tail length of 60 mg/kg treated groups showed a highly significant ($p \leq 0.001$) increase as compared to control (Figure 4). A highly significant increase ($p \leq 0.001$) in percentage DNA in comet tail of 60 mg/kg was observed compared to control. The other group showed a significant increase ($p < 0.05$) as compared to control. However after 20 days of withdrawal of treatment no recovery to normal levels was observed in both the treated groups (Table 2 and Figure 5).

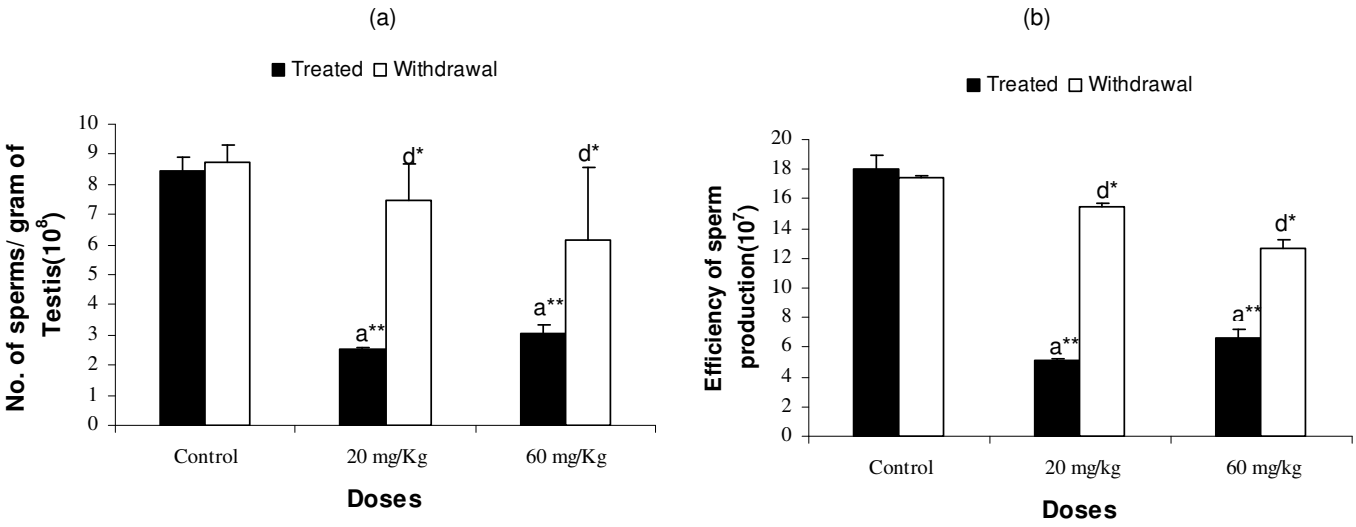
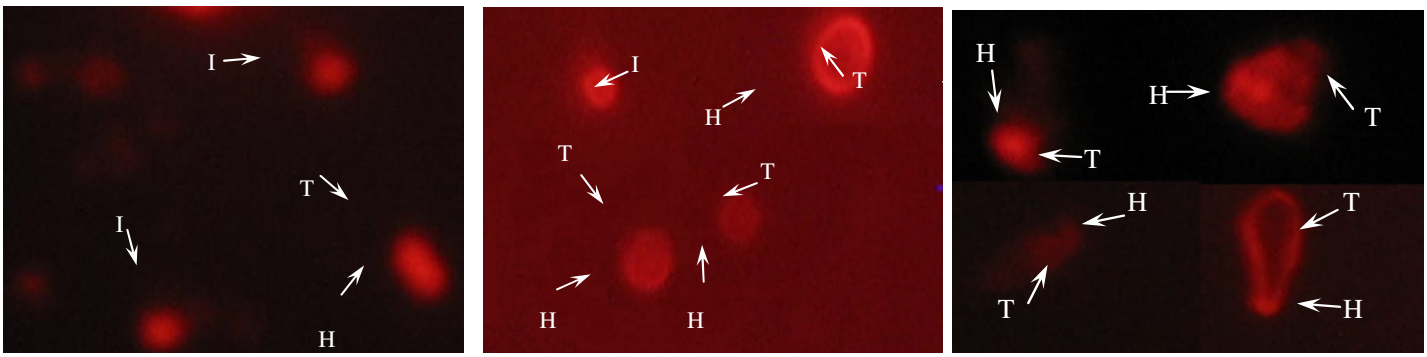


Figure 2. Mean (a) sperm count per gram of testis (b) efficiency of sperm production in control, *A. precatorius* treated male albino mice and after 20 days of withdrawal of treatment. Values expressed as Mean ± SEM, a = control vs. treatment groups (20 mg/kg, 60 mg/kg), d = Treated vs. Withdrawal, **p≤0.001, *p<0.05

Table 2. Mean comet number, length, height, area, tail length, tail moment, % DNA in tail and olive moment in control and *A. precatorius* treated adult male albino mice.

Parameters	Control		20 mg/kg		60 mg/kg	
	Treated	Withdrawal	Treated	Withdrawal	Treated	Withdrawal
Comet number (150 Cells)	64.50 ± 0.50	60.00 ± 6.00	74.00 ± 1.00^{a*}	72.50 ± 2.50^{a*}	100.00 ± 2.00^{a**,b**}	105.00 ± 2.00^{a**,b**}
Comet length (µm)	3.38 ± 0.34	6.63 ± 0.19	6.84 ± 0.43^{a**}	4.84 ± 0.14^{a**}	6.98 ± 0.55^{a**}	6.98 ± 0.52^{a**,b**}
Comet height (µm)	3.07 ± 0.41	2.27 ± 0.13	5.60 ± 0.38^{a**}	3.34 ± 0.12^{a**}	5.64 ± 0.45^{a**}	4.88 ± 0.28$^{a**, b**}$
Comet area (µm^2)	6.11 ± 0.30	6.37 ± 0.85	39.58 ± 4.54^{a**}	16.19 ± 0.81^{a*}	43.82 ± 6.04^{a**}	37.57 ± 4.48$^{a**, b**}$
Tail length (µm)	0.44 ± 0.09	0.45 ± 0.09	1.72 ± 0.28^{a*}	1.52 ± 0.16^{a*}	2.51 ± 0.34^{a**}	2.41 ± 0.36^{a**}
Tail moment	1.41 ± 0.41	0.99 ± 0.21	1.50 ± 0.39	1.58 ± 0.33	2.02 ± 0.54	2.01 ± 0.44
% DNA in tail	5.67 ± 1.37	4.60 ± 1.47	11.73 ± 1.78^{a*}	6.99 ± 2.12	14.57 ± 1.56^{a**}	15.25 ± 3.49^{a*}
Olive moment	1.38 ± 0.14	1.13 ± 0.27	3.04 ± 0.71	2.91 ± 0.56^{a*}	3.91 ± 0.54^{a*}	3.24 ± 0.42^{a**}



(a) (b) (c)

Figure 3. Fluorescent photomicrograph (×400) of adult mice sperm DNA after A. precatorius treatment using comet assay, stained with acridine orange, showing (a) control with more intact DNA sperms, (b) few comets with short tails in 20 mg/kg treated and (c) more comets with long tails in 60 mg/kg treated mice. I = Intact cell, H = Head of the comet and T = Tail of the comet.

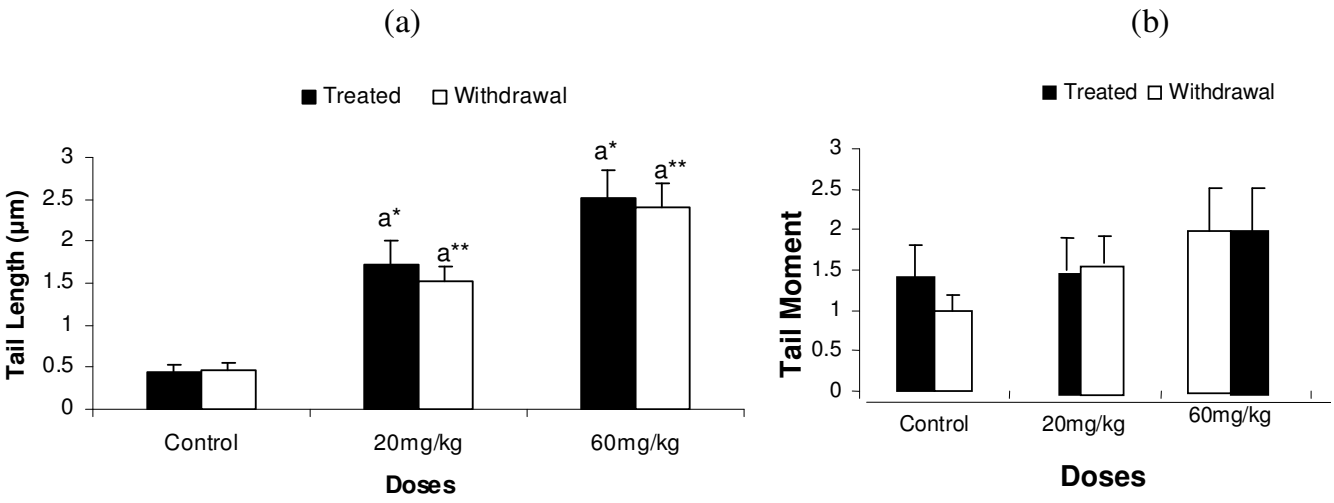


Figure 4. (a) Comet tail length and (b) tail moment in control and *A. precatorius* treated adult male albino mice. Values expressed as Mean ± SEM, a = control vs. treatment groups (20 and 60 mg/kg), **p≤0.001, *p<0.05.

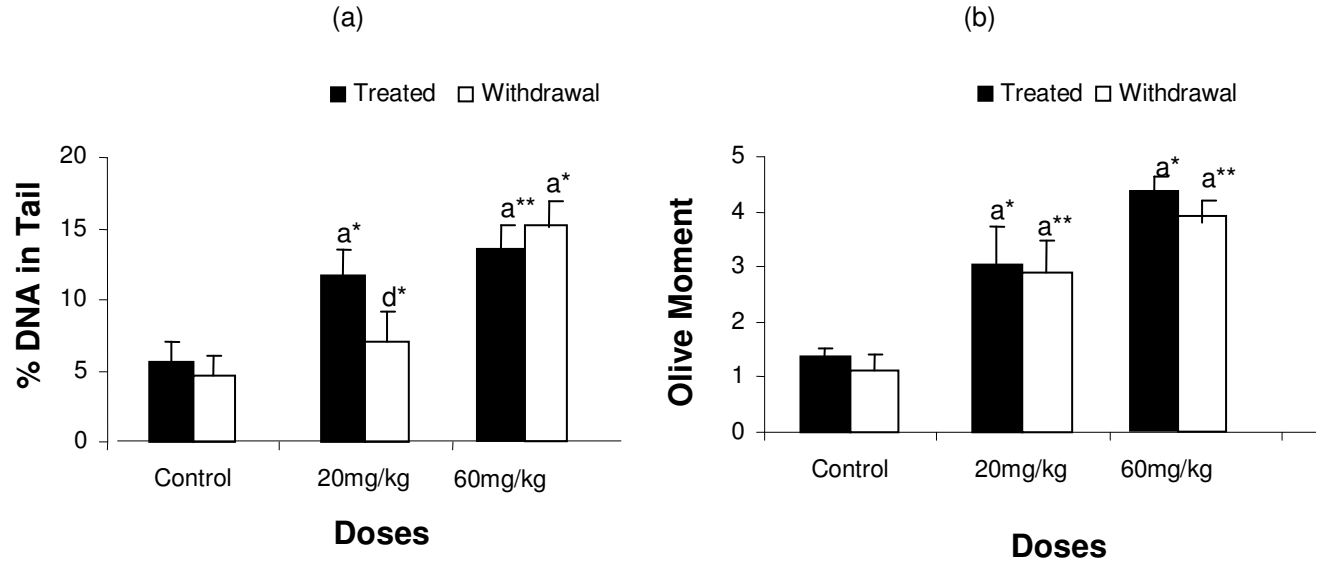


Figure 5. (a) % DNA in comet tail (b) comet olive moment in control and *A. precatorius* treated adult male albino mice. Values expressed as Mean ± SEM, a = control vs. treatment groups (20 and 60 mg/kg), d = Treated vs. Withdrawal, **p≤0.001,*p<0.05.

DISCUSSION

The present study also suggested that an intraperitoneal administration of ethanolic seed extract of *A. precatorius* causes a dose independent reduction in sperm production while spermatozoa DNA damage is dose dependent. Present investigation shows a significant decrease in number of spermatids in testes after administration of seed extract of *A. precatorius* which is in agreement with previous study (Rao, 1987). A decline in caudal sperm motility and count followed by viability reduction in extract fed rats, further, scanning electron microscopic study on sperm morphology exhibited decapitation, acrosome damage and formation of bulges on midpiece region of sperms in A. precatorius (100 mg/kg) treated rats. (Sinha and Mathurm, 1990) reported suppressed sperm motility in caudal epididymis as the most pronounced effect of treatment of 50% ethanol extract of *A. precatorius* seeds (250 mg/kg) in 30 and 60 days albino rats which declined the fertility rate. An inhibitory effect of a methanolic seed extract of *A. precatorius* on motility of washed human spermatozoa has also been reported (Ratnasooriya et al., 1991).

Bhatt et al. (2007) reported that *A. precatorius* has reversible antifertility or antispermatogenic effect in mice but present study demonstrated a significant increase in daily sperm production after 20 days of withdrawal of

treatment suggesting an effective contraceptive activity of seed extract of A. precatorius. Abrin may induce infertility by causing inactivating of rRNA thus resulting in inhibition of protein synthesis in sertoli and leydig cells (Bagaria et al., 2006) or it may directly interact with the mitochondrial membrane of spermatids causing apoptosis of spermatids (Bhutia et al., 2008), while steroids can cause infertility by replacing the natural steroids that is, LH, FSH and testosterone thus inibiting the process of spermatogenesis (Sinha and Mhathurm, 1990).

Evaluation of DNA damage by seed extract of *A. precatorius* was made by comet assay. In contrast to sperm production there was a dose dependent increase in number of spermatozoa with damaged DNA. The low dose caused a significant increase ($p < 0.05$) DNA damage compared to control while high dose induced a highly significant increase ($p \leq 0.001$) in DNA damage compared to control. The intensity of DNA damage is in agreement with the previous studies which indicate DNA fragmentation caused by isolated abrin from the seeds of A. precatorius (Shih et al., 2001; Qu and Qing, 2004).

In this experiment the treatment was withdrawn for 20 days. After 20 days of withdrawal of treatment, there was a non significant increase in the number of spermatozoa with damaged DNA in groups treated with high dose. At low dose there was a non significant decrease in the number of spermatozoa with damaged DNA suggesting requirement of a long time span after withdrawal of treatment for DNA repair. So the present work suggests that the low doses of seed extract of A. precatorius have a greater tendency to retain DNA integrity as compared to those receiving higher doses. It can also be inferred by the present study that the seed extract of A. precatorius has a capability to induce DNA damage for sometime even after withdrawal of treatment. The best possible mechanism for the dose dependent effect of seed extract of A. precatorius on DNA integrity may involve production of reactive oxygen species by interaction of toxic proteins of seeds of A. precatorius that is, abrin and agglutinin with the antioxidant proteins of the cell as shown in previous studies (Shih et al., 2001; Qu and Qing, 2004; Bagaria et al., 2006). It has also been determined that agglutinin protein can induce apoptosis by interacting with mitochondrial membrane and thus inactivating mitochondria (Narayanan et al., 2004; Stirpe and Batteli, 2006; Bhutia et al., 2008) which is the most important organelle of the cell especially in spermatozoa it has a very crucial role in motility as well as survival of sperms. Involvement of other components of seeds of A. precatorius in DNA damage remains to be revealed.

Conclusion

Present investigation indicates that the role of seed extract of A. precatorius as a contraceptive or antifertility agent with a risk of DNA damage or genotoxicity in spermatozoa and may lead to teratogenic effects. In the light of present study, further investigations are required with a focus on the isolation of such bioactive constituents of the seeds of A. precatorius which have ability to induce contraceptive activity without any genotoxic effects or DNA damage in spermatozoa.

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